DESIGN AND EVALUATION OF CHITOSAN AND *N*-TRIMETHYL CHITOSAN CHLORIDE MICROSPHERES FOR INTESTINAL DRUG DELIVERY

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"Learn from yesterday, live for today, hope for tomorrow. The important thing is not to stop questioning."

Albert Einstein

To my parents

Carl and Petro Venter

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ABSTRACT

The absorption enhancing ability of chitosan, a linear polysaccharide, is mediated by protonated amino groups on the C-2 position of the molecules that induce interaction with the anionic sites on the cell membranes to subsequently alter tight junction integrity. In neutral and basic environments, such as those found in the small and large intestines, most chitosan molecules will lose their charge and precipitate from solution rendering it ineffective as an absorption enhancer. To increase the solubility of this polymer, methylation of the amino groups on the C-2 position was proposed.

A partially quaternised and water soluble derivative of chitosan, *N*-trimethyl chitosan chloride (TMC), which exhibits superior solubility in a basic environment compared with other chitosan salts was synthesised and included in a chitosan microbead solid drug delivery system. Two TMC derivatives were synthesised by reductive methylation from high and medium molecular weight Chitoclear™ chitosan respectively. The degree of quaternisation calculated from the ^{1}H-NMR spectra for the medium molecular weight TMC (TMC-M) and the high molecular weight TMC (TMC-H) polymers were 74.7 % and 48.5 % respectively. The mean molecular weights of the synthesised TMC-M and TMC-H polymers were 64 100 g/mole and 233 700 g/mole respectively. The effect of different concentrations TMC-M and TMC-H on chitosan microbeads was studied with results obtained from scanning electron microscopy (SEM), TMC loading capacity and microbead swelling behaviour. After selection of the most suitable TMC concentration, the effect of varying concentration (0.1, 0.2 and 0.5 %) additives on TMC and ibuprofen release was studied. Commonly used modified cellulose gum (Ac-di-sol® (ADS)), sodium starch glycolate (Explotab® (EXP)) and ascorbic acid (AA) were added as disintegrants to different microbead formulations to promote release of both the ibuprofen as model drug and TMC from the beads. It was noticed that the loading (% drug loading capacity) of TMC-M was much lower

than that obtained with TMC-H while the inclusion of different additives in varying concentrations did not seem to have a profound influence on the loading of either TMC-M or TMC-H. It was further noticed from the fit factors (f_1 and f_2) for dissolution profiles of eighteen chitosan microbead variations that the formulation containing TMC-H and 0.5 % (w/v) ascorbic acid was the only formulation with a significantly higher ibuprofen and TMC-H release profile compared to all other formulations tested.

The chitosan microbead formulation containing 2 % (w/v) TMC-H and 0.5 % (w/v) ascorbic acid (H-AA-0.5) was used for *in vitro* absorption studies through rat intestine in Sweetana-Grass diffusion chambers. Chitosan containing TMC-H (no ascorbic acid) (CHIT-H) only and a plain chitosan microbead (CHIT) formulation was used as control formulations during the *in vitro* studies. Although the H-AA-0.5 formulation exhibited the highest transport rate for ibuprofen, the mean rate of transport (P_{app}) obtained from the two formulations containing TMC-H (CHIT-H and H-AA-0.5) showed no significant difference in the transport rate of ibuprofen. Compared to the CHIT formulation as control, both formulations containing TMC-H exhibited increased ibuprofen transport across *in vitro* rat jejunum. However, a statistical significant increase in transport was obtained only from the H-AA-0.5 formulation in comparison with the CHIT formulation.

It can be concluded that the combination of high molecular weight TMC with a low degree of quaternisation and ascorbic acid (0.5 % w/v) in a chitosan microbead lead to a statistical significant increase in the *in vitro* transport rate of ibuprofen through rat jejunum.

Keywords: Absorption enhancement; Quaternised chitosan; Mucoadhesion; Microbeads; Ibuprofen; Sweetana-Grass diffusion chambers.

UITTREKSEL

Die absorpsiebevorderende eienskappe van kitosaan, 'n liniêre polisakkaried, word veroorsaak deur die gepronotoneerde aminogroepe op die C-2 posisie van die molekule. Hierdie groepe induseer 'n interaksie met die anioniese gedeeltes op die selmembraan om daardeur die digsluitende hegtingskomplekse se funksie te wysig. In neutrale en basiese omgewings, soos die van die dun- en dikderm, verloor die kitosaan molekule sy lading en presipiteer uit oplossing en is daarom oneffektief as 'n absorpsiebevorderaar. Om die oplosbaarheid van hierdie polimeer te verbeter is metilering van die aminogroepe op die C-2 posisie van die polimeer voorgestel.

Gedeeltelik gekwaterniseerde en goed wateroplosbare derivate van kitosaan, *N*-trimetiel kitosaan chloride (TMC), is in hierdie studie gesintetiseer en in 'n kitosaan mikrokorreltjie ingesluit wat as soliede geneesmiddel afleweringsisteem dien. Reduserende metilering is gebruik om twee TMC derivate vanaf onderskeidelik hoë en medium molekulêre gewig Chitoclear™ kitosaan te sintetiseer. Vanaf die ^{1}H-KMR spektra is die graad van kwaternisering vir medium molekulêre gewig TMC (TMC-M) en hoë molekulêre gewig TMC (TMC-H) bereken as onderskeidelik 74.7 % en 48.5 %. Die gemiddelde molekulêre gewigte vir die gesintetiseerde TMC-M en TMC-H polimere was onderskeidelik 64 100 g/mol en 233 700 g/mol. Die effek van verskillende konsentrasies TMC-M en TMC-H op kitosaan mikrokorreltjies is met behulp van elektronmikroskopie (SEM), TMC ladingskapasiteit en mikrokorreltjie swellingsgedrag bestudeer. Die effek op TMC en ibuprofen vrystelling deur 'n variasie in die konsentrasie (0.1, 0.2 en 0.5 %) van verskillende bymiddels is verder bestudeer nadat die geskikste konsentrasie TMC bepaal is. Algemeen gebruikte sellulose gom (Ac-di-sol® (ADS)), natrium stysel glikolaat (Explotab® (EXP)) en askorbiensuur (AA) is as disintegreermiddels bygevoeg in verskillende mikrokorreltjie formulerings om die vrystelling van beide ibuprofen as model geneesmiddel en TMC te bevorder. Die

lading (% geneesmiddel ladings kapasiteit) van TMC-M was heelwat laer as die lading van TMC-H, terwyl die insluiting van die verskillende bymiddels in verskillende konsentrasies geen noemenswaardige invloed op die lading van beide TMC-M en TMC-H gehad het nie. Die passingsfaktore (f_1 en f_2) vir die dissolusie profiele van die agtien kitosaan mikrokorreltjie variasies het verder aangedui dat die formule met TMC-H en 0.5 % (m/v) askorbiensuur betekenisvolle hoër ibuprofen en TMC-H vrystelling getoon het in vergelyking met al die ander mikrokorreltjie variasies.

Die kitosaan mikrokorreltjies wat 2 % (m/v) TMC-H en 0.5 % (m/v) askorbiensuur (H-AA-0.5) bevat is verder gebruik vir *in vitro* absorpsie studies in Sweetana-Grass diffusie kamers. Kitosaan mikrokorreltjies wat slegs TMC-H (geen askorbiensuur) (CHIT-H) bevat en gewone kitosaan mikrokorreltjies (CHIT) is as kontroles vir die *in vitro* studies gebruik. Alhoewel die H-AA-0.5 formule die hoogste transport van ibuprofen getoon het, het die gemiddelde tempo van transport (P_{app}) wat vir beide die TMC- H bevattende formules (CHIT-H en H-AA-0.5) bereken is getoon dat geen noemenswaardige verskil in die tempo van ibuprofen transport plaasgevind het nie. In vergelyking met die CHIT formule as kontrole, het beide die TMC-H bevattende formules 'n verhoogde ibuprofen transport getoon. 'n Statisties betekenisvolle verhoging in ibuprofen transport is egter slegs verkry met die H-AA-0.5 formule wanneer dit met die CHIT kontrole vergelyk word.

Opsommend is gevind dat die kombinasie van hoë molekulêre gewig TMC, wat oor 'n lae graad van kwaternisering beskik, en askorbiensuur (0.5 % m/v) in kitosaan mirokorreltjies lei tot 'n statisties betekenisvolle verhoging in die *in vitro* transport tempo van ibuprofen deur rot jejunum.

Sleutelwoorde: Absorpsiebevordering; Gekwaterniseerde kitosaan; Mukoklewendheid; Mikrokorreltjies; Ibuprofen; Sweetana-Grass diffusie kamers.

INTRODUCTION AND AIM OF THE STUDY

Over the past 25 years, interaction among the fields of polymer and material science and the pharmaceutical industry has resulted in the development of what are known as drug delivery systems (DDS). To maximise the efficacy and safety of medicines, DDS should provide a desired rate of delivery of the therapeutic dose, at the most appropriate area in the body, in order to prolong the duration of pharmacological action and reduce the adverse effects.

The oral route is the route of first choice for drug administration and allows the attainment of systemic effects of a large variety of biologically active compounds. However, on oral administration various drugs exhibit relatively low bioavailability. This may be caused by precipitation or binding of the drug in the gastrointestinal tract, degradation in the gastrointestinal lumen or by extensive first-pass metabolism. Furthermore, the intestinal epithelium presents the major barrier to absorption of orally administered drugs into the systemic circulation. In order to overcome the absorption barrier, permeation enhancers are used as auxiliary agents in oral drug delivery systems.

Permeation enhancers, substances that facilitate the transport of solutes across biological membranes, have been investigated in that capacity for at least five decades. The potential use of chitosan, and the partially quaternised derivative *N*-trimethyl chitosan chloride (TMC), as absorption enhancers across mucosal surfaces has been well documented in recent years (Illum *et al.*, 1994:1186; Artursson *et al.*, 1994:1358; Domard *et al.*, 1986:105; Kotzé *et al.*, 1999a:341). Chitosan is also a polycationic polymer, well known for its chelating properties (Berger *et al.*, 2004:27). Therefore, reactions with negatively charged components, either ions or molecules, can lead to the formation of a network through ionic bridges between polymeric chains. Using innovative

microencapsulation technologies, and by varying the copolymer ratio and the molecular weight of the polymer, etc., chitosan microbeads can be developed into an optimal drug delivery system for a particular drug. Previous studies performed by Lubbe (2002:49) showed poor release of TMC from freeze-dried chitosan beads with subsequent reduced permeation enhancing properties. The results were attributed to:

- poor solubility of the synthesised TMC polymer at the experimental pH 6.8,
- low loading capacity of TMC into chitosan beads, possibly due to poor entanglement of short TMC chains with the chitosan network,
- synthesis of relatively large beads (≥ 2000 µm) which lead to a decreased adsorption surface area.

Since TMC acts as a useful absorption enhancer it must be released from the beads to cause improved potential bioavailability. It was hypotesised that the inclusion of disintegrating agents, as well as TMC polymers with different degrees of quaternisation and different molecular weights, into chitosan microbeads might enhance the release of TMC from the beads to act as permeation enhancer. It was further hypothesised that a reduction in the size of chitosan beads might lead to increased permeation enhancement due to an increase in the adsorption surface area. The specific objectives of this study were to:

1. Conduct a literature study on the role of polymers as absorption enhancers to determine the suitability of chitosan and TMC as possible absorption enhancers.
2. Conduct a literature study on chitosan microspheres as drug delivery devices to select the most feasible method for producing chitosan microbeads to act as drug delivery systems.

3. Synthesise and characterise both a high molecular weight (TMC-H) and medium molecular weight (TMC-M) *N*-trimethyl chitosan chloride polymer with different degrees of quaternisation.
4. Prepare and characterise chitosan microbeads containing one of the synthesised TMC polymers, a disintegrating agent (Ac-di-sol®, Explotab® or ascorbic acid) and ibuprofen as model drug. These studies were performed to determine the following:

    - if chitosan beads are indeed a suitable carrier for TMC and ibuprofen,
    - which TMC derivative (TMC-M or TMC-H) is the best candidate for loading into and release from chitosan microbeads, and
    - which concentration of additive (Ac-di-sol®, Explotab® or ascorbic acid) shows the most favourable release of both TMC and ibuprofen.

5. Develop a HPLC analytical method that was easy to use and sensitive enough for the quantitative determination of ibuprofen in phosphate buffered solution (PBS), following permeation through rat jejunum.
6. Perform *in vitro* permeability/transport studies of ibuprofen from the most promising chitosan microbead formulation across rat intestine using a vertical diffusion chamber system.

Chapter 1 will provide more information on polymers as absorption enhancers and explain the physiology of the human gastrointestinal tract, while chapter 2 will focus on the feasibility of chitosan microspheres as drug delivery devices. In chapter 3 the synthesis and characterisation of the two TMC polymers obtained are documented. Chapter 4 will focus on the preparation and characterisation of different variants of chitosan microbeads containing ibuprofen, one of the synthesised TMC polymers and a disintegrating agent (Ac-di-sol®, Explotab® or ascorbic acid). In chapter 5 the most promising chitosan microbead variant were used for *in vitro* permeability/transport studies of ibuprofen across rat intestine.

CHAPTER 1

POLYMERS AS ABSORPTION ENHANCERS OF HYDROPHILIC DRUGS ACROSS INTESTINAL EPITHELIA

For most of civilised history, there was no clear difference in the way in which humans consumed food and medicine. To date, oral delivery is still the preferred route of drug administration, especially for chronic therapies where repeated administration is required. Oral administration offers patients less pain, greater convenience, higher likelihood of compliance, and reduced risk of cross-infection and needle stick injuries (Chen and Langer, 1998:340). Despite these advantages, the oral route has a high susceptibility to digestive enzymes in the gastrointestinal (GI) tract, poor absorption and a limited ability for transport across the intestinal epithelial barrier. In the passed few years, a number of significant advances have been made in the development of new technologies for optimising oral drug delivery.

To maximise the efficacy and safety of medicines, drug delivery systems (DDS) should provide a desired rate of delivery of the therapeutic dose, at the most appropriate area in the body, in order to prolong the duration of pharmacological action and reduce the adverse effects, minimise the dosing frequency and enhance patient compliance. In most cases, the oral bioavailability of hydrophilic macromolecules is strongly limited by an insufficient uptake from the mucosa. In order to overcome the absorption barrier, permeation enhancers are used as auxiliary agents in oral drug delivery systems. Most of these permeation enhancers, however, are of low molecular mass and thus being much more rapidly absorbed from the intestine than the drug itself (Aungst, 2000:430). In addition, systemic toxic side effects of these auxiliary agents cannot be excluded. One alternative class of permeation enhancers that has received lots of attention in order to overcome these shortcomings are high molecular mass polymers

such as polyacrylates or chitosans (Borchard *et al.*, 1996:131). Polysaccharides are polymers of monosaccharides (sugars). They are found in abundance, have wide availability, they are inexpensive and available in a variety of structures with a variety of properties (Hovgaard and Brondsted, 1996:185). They can be easily modified chemically and biochemically and are highly stable, safe, non-toxic, hydrophilic and gel forming and in addition biodegradable, which suggests their use in DDS. They display certain advantages in comparison to low molecular mass enhancers like additional mucoadhesive properties, which allow them to remain concentrated at the area of drug absorption (Lehr, 1996:140). Consequently, a steep concentration gradient representing the driving force for passive drug uptake is likewise provided for therapeutic agents being embedded in the mucoadhesive polymeric carrier matrix. As these polymers will not be absorbed from the gut due to their high molecular mass (Bar *et al.*, 1995:911), systemic side effects can be excluded and a prolonged permeation enhancing effect is provided. Large numbers of polysaccharides, such as chitosan, pectin, chondroitin sulphate, cyclodextrins, dextrans, guar gum, pectin, locust bean gum and amylose have already been investigated for their potential as drug delivery systems.

1.1 PHYSIOLOGY OF THE HUMAN GASTROINTESTINAL TRACT

The gastrointestinal (GI) tract, also called the alimentary canal, is a muscular digestive tube that winds through the body. It functions as a selective barrier between the environment and the systemic circulation, which functions to digest dietary food, to absorb nutrients, electrolytes and fluid, and to prevent the absorption of potentially harmful substances. The GI system is differentiated into organs which possess unique characteristics along its length (Kutchai, 1998:589). Figure 1.1 shows a summary of the entire alimentary tract. Each part is adapted to its specific functions: some the simple passage of food, such as the oesophagus; others the storage of food, such as the stomach; and others the digestion and absorption of food, such as the small intestine (Fox, 1996:541).

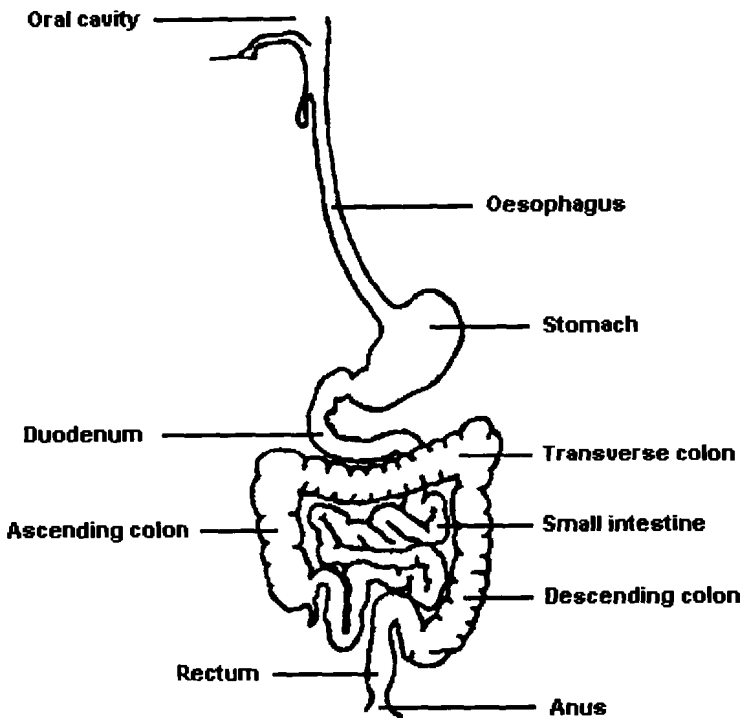


FIGURE 1.1 Schematic summary of the gastrointestinal tract (adapted from Fox, 1996:541).

1.1.1 The stomach

The stomach is situated in the left upper part of the abdominal cavity immediately under the diaphragm. Its size varies according to the amount of distension: up to 1500 ml following a meal; after food has emptied, a 'collapsed' state is obtained with a resting volume of only 25 to 50 ml (Guyton and Hall, 1996:806). The stomach is composed of the following parts: (1) fundus, above the opening of the oesophagus into the stomach; (2) body, the central part; and (3) antrum. The pylorus is an anatomical sphincter situated between the most terminal antrum and the duodenum. The fundus and the body store food temporarily, secrete digestive juices and propel chyme, a milky mixture of food with gastric juices, to the antrum. The antrum grinds and triturates food particles and regulates the secretion of hydrochloric acid as well as the emptying of food (Kutchai, 1998:603). Fasting gastric pH is usually steady and approximates 2 to 6, but in

the presence of food the pH is approximately 1.5 to 2. This strong acidity serves three functions: (1) ingested proteins are denatured at low pH – that is, their tertiary structure is altered so that they become more digestible; (2) under acidic conditions, weak pepsinogen enzymes partially digest each other – this frees the active pepsin enzyme as small peptide fragments are removed; and (3) pepsin is more active under acidic conditions (optimum pH of about 2.0). Proteins are partially digested by the action of pepsin, while carbohydrates and fats are not digested at all in the stomach. The complete digestion of food molecules occurs later, when chyme enters the small intestine (Fox, 1996:546). It is noticeable that the stomach provides a very hostile environment towards all of its contents with the main purpose of processing and storing.

1.1.2 The small intestine

The small intestine is the longest part of the GI tract and the site, particularly the duodenum and jejunum, where most digestion and absorption take place. The first 5 % or so of the small intestine is the duodenum with a pH of 6.1 and a relatively short transit time, less than 1 min (Guyton and Hall, 1996:808). The remaining small intestine is divided into the jejunum and the ileum. The pH of this part of the small intestine is 6 to 7 and the transit time of 3 ± 1 hours is relatively constant and unaffected by food (Kutchai, 1998:607). The mucosa of the small intestine is folded into villi that project into the lumen. In addition, the cells that line these villi have folds of their plasma membrane called microvilli. This arrangement greatly increases the surface area for absorption, which is comparable to the area of a basketball court, 463 m^2 (Read and Sugden, 1987:222). This is the main reason why it is considered as the primary absorption site of water, ions, vitamins and nutrients such as amino acids, fats and sugars. In addition, the digestion of fats, peptides and sugars occurs in this segment of the gastrointestinal tract, since the digestive enzymes of the small intestine are embedded within the cell membrane of the microvilli (Fox, 1996:549).

1.1.3 The large intestine

The large intestine is the last major subdivision of the GI tract. The digested materials that reach the large intestine contain few nutrients, but the residues remain here for 12 to 24 hours. The pH is 6.4 in the ascending colon, rises in the transverse colon, and approaches neutrality in the descending colon. In the colon mainly water, ions, certain drugs and especially peptide molecules are absorbed. This is despite the lack of villi, which leads to small surface area (Fox, 1996:552).

1.2 INTESTINAL ABSORPTION BARRIERS FOR HYDROPHILIC DRUGS

Before a compound is transferred from the intestinal lumen to the blood it has to pass several absorption barriers. The potential physical barriers for intestinal drug absorption are located in the unstirred water layer, the mucus layer, the apical and basolateral cell membranes and cell contents, the basement membrane, the tight junctions and the wall of lymph and blood capillaries (figure 1.2) (Van Hoogdalem *et al.*, 1989:409). The intestinal epithelium presents the major barrier to absorption of orally administered drugs into the systemic circulation (Hochman and Artursson, 1994:253). In order to overcome the gastrointestinal absorption barriers, hydrophilic molecules have to pass the epithelium via the epithelial cell (transcellular) or between cells via the tight junctions and intercellular space (paracellular) (Van Hoogdalem *et al.*, 1989:408). Whether the administered compounds will be transported through the transcellular or paracellular route will be judged by the physical and chemical properties of the drug. Highly lipophilic compounds diffuse passively across the membrane via the transcellular pathway as this transport requires partitioning of the compound through both the apical and the basolateral membranes.

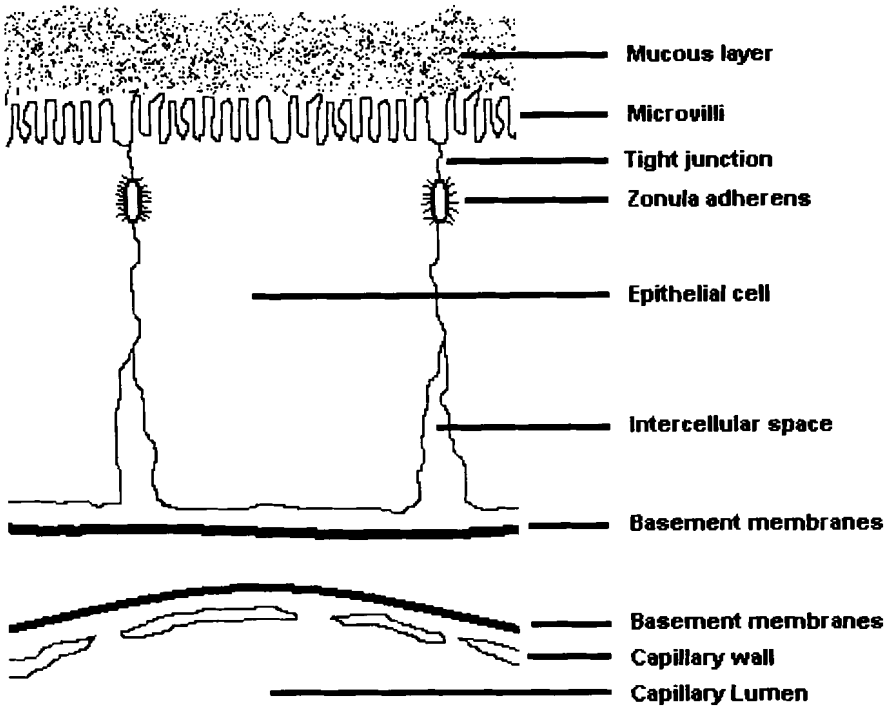


FIGURE 1.2 Schematic representation of the intestinal epithelial cell (adapted from Van Hoogdalem *et al.*, 1989:409).

Consequently with the exception of those compounds which are transported by active or facilitated mechanisms, absorption of larger membrane-impermeable and more hydrophilic drugs, diffuse to a higher extent through the paracellular pathway (Hochman and Artursson, 1994:253). Although either the transcellular or paracellular route can be the favoured way of mucosal uptake, in most cases both routes are involved in the absorption process (Muranishi, 1990:8). Conceptually, the phospholipid bilayer of the plasma membrane is considered to be the major factor restricting the free movement of substances from the lumen to the bloodstream through the transcellular pathway (Van Hoogdalem *et al.*, 1989:409). For the paracellular route of absorption, which is strictly based on a passive diffusion process, it was demonstrated that molecules with a radius above 15 Å can hardly pass the tight junctions representing the limiting gate fence area for the paracellular pathway (Cereijido *et al.*, 1981:97; Madara and

Dharmasathaphorn, 1985:2126). Thus considerable attention has been directed at finding ways to increase paracellular transport by 'loosening' tight junctions.

1.2.1 Morphology of the tight junctions

Tight junctions (zonulae occludentes) are regions of close contact between apical ends of epithelial cells. In transmission electron microscopy (TEM), the tight junction appears as an approximately 80 nm long region at the boundary of neighbouring cells in which the plasma membranes of adjacent cells are brought into close apposition. Within this structure 'kiss' sites are apparent in which the neighbouring plasma membranes come into close intimate contact. These sites presumably represent structures which confer most of the restriction to diffusion across the tight junction (Hochman and Artursson, 1994:255). They are constructed of a meshwork of strands, the tight junction permeability increasing with decreasing strand number, thus determining the 'leakiness' of the epithelium. The small intestine contains a relatively leaky epithelium and the intestinal permeability decreases in the distal direction. The proximal colon is moderately leaky and the distal colon is moderately tight (Van Hoogdalem *et al.*, 1989:411).

In order to understand the influence of tight junctions on the paracellular absorption, it is useful to be aware of the proteins regulating and/or influencing the gate fence function of the tight junctions. In figure 1.3, the most likely important proteins building up the tight junctions are presented. A family of proteins being located in the region of tight junctions is the claudins. The originally identified proteins are claudin-1 and -2 with a molecular mass of 22 to 24 kDa expressing two extracellular loops (Furuse *et al.*, 1998:1546). At least some of the claudins are able to mediate cell adhesion in a Ca^{2+}-independent manner (Kubota *et al.*, 1999:1036). Their function is seen in the selection of ions passing through the paracellular barrier (Simon *et al.*, 1999:104). The junctional adhesion molecule (JAM) is another protein found in the tight junctions, being a

member of the immunoglobulin superfamily, and thus structurally very distinct from the claudins. The influence of JAM on tight junctional integrity is not completely understood, but strong evidence is given that it may play an additional role in cell-cell adhesion (Martin-Padura *et al.*, 1998:119).

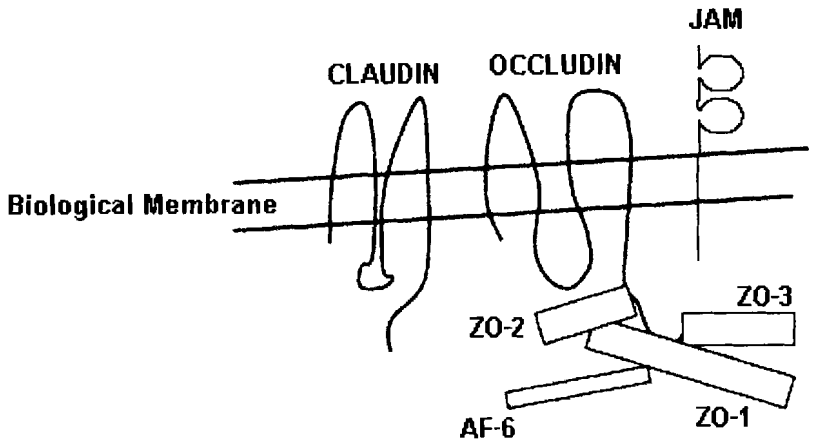


FIGURE 1.3 Molecular components of the tight junctions: AF-6 = protein containing one domain being able to interact with ZO proteins and two domains that can disrupt this interaction; ZO-1, ZO-2, ZO-3 = zona occludens proteins 1, 2, 3; JAM = junctional adhesion molecule (Furuse *et al.*, 1998:1546).

The third known transmembrane protein at the tight junctions is occludin. It is a 60 to 65 kDa protein that was shown to express two extracellular loops from amino acid 81 to 124 and 184 to 227. These loops express several tyrosine and glycine residues, as shown in figure 1.4, and are believed to provide the cohesiveness of the junctional barrier (Furuse *et al.*, 1998:1546).

1.2.2 Regulation of the tight junctions

A number of agents which disrupt tight junction regulation have been identified and understanding the mechanisms underlying the tight junction disruption may provide clues to novel approaches to promoting drug absorption. Two factors

which could underlie these mechanisms are contraction of the perijunctional actin-myosin ring and protein kinase- or phosphatase-mediated changes in tight junction protein phosphorylation (Hochman and Artursson, 1994:257).

Adjacent to the tight junction in the cytoplasm is an actin-myosin ring which circumscribes the cell. This ring is associated with the plasma membrane and can contract, exerting an inward force on the lateral plasma membrane. Such contractions have been correlated with a loosening of the tight junction, indicating that contractions of the perijunctional ring pull on tight junction components, inducing separations in the junctional complexes of neighbouring cells. Further evidence for a direct link between this perijunctional actin-myosin ring and tight junctions has been inferred by: (1) direct observation of tight junction associated actin and (2) by observations showing disruption in the structure and integrity of tight junctions by agents that disrupt actin filaments (Hochman and Artursson, 1994:257).

Phosphorylation of tight junction components may also be an important mediator in regulation of tight junction integrity. Tyrosine residues are phosphorylated by protein tyrosine kinases resulting in increased tight junctional permeability as shown in figure 1.4 (Collares-Buzato *et al.*, 1998:88). Protein tyrosine phosphatases (PTP), on the other hand, are able to dephosphorylate these groups, which were shown to result in a closing of the tight junctions (Collares-Buzato *et al.*, 1998:92). Consequently, inhibition of PTP leads to more phosphorylated occludin and to more open tight junctions. As PTP bears an active site, a cysteine moiety, being responsible for its activity, glutathione (GSH) was shown to be capable of inhibiting PTP activity by almost 100 % via a disulfide bond formation with this cysteine substructure within minutes (Barrett *et al.*, 1999:6701). Being aware of this high inhibitory effect of GSH towards PTP, high concentrations of GSH should lead to more open tight junctions. So far, however, it was only demonstrated that GSH is involved in a H_2O_2-mediated increase in paracellular permeability in Caco-2 (human colon carcinoma) cell monolayers (Rao *et al.*, 2000:333). Recent studies showed that the permeation

enhancing effect of GSH on freshly excised intestinal mucosa is not convincing (Clausen *et al.*, 2002:603).

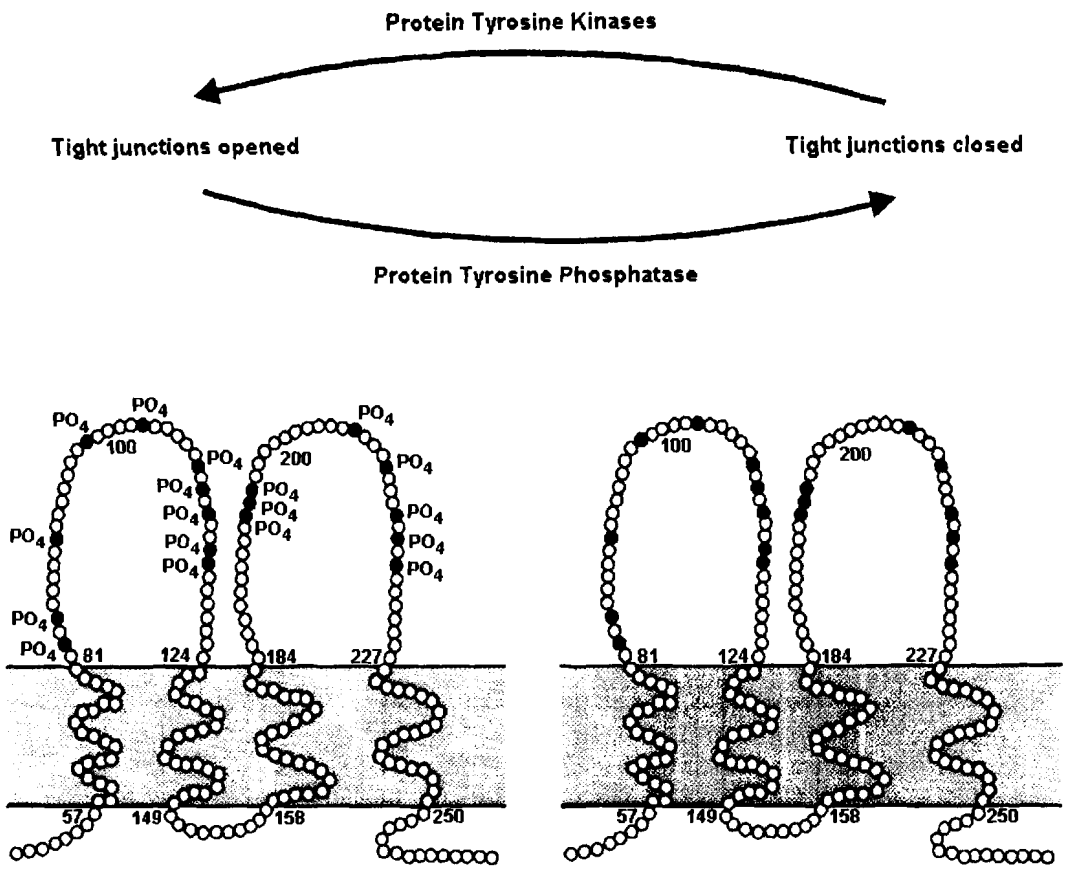


FIGURE 1.4 Schematic representation of enzymes controlling the degree of phosphorylation of the tyrosine subunits of occluding (Furuse *et al.*, 1998:1546).

An explanation for these observations might be given by the rapid oxidation of GSH on the cell surface (Grafstrom *et al.*, 1980:571), whereas other stable PTP inhibitors, such as phenylarsine oxide or pervanadate, were shown to strongly increase tight junction permeability (Staddon *et al.*, 1995:610). Thus modulation of the phosphorylation state of tight junction components may be one means for regulating junctional integrity without inducing disassembly of the junctional complex.

1.3 POLYMERS AS MUCOSAL ABSORPTION ENHANCERS

In order to increase the absorption of poorly permeable drugs, excipients such as absorption enhancers have been evaluated. Absorption enhancers facilitate the absorption of solutes across biological membranes (transcellular transport) and through pores between adjacent epithelial cells (paracellular transport) as mentioned in § 1.2. The ideal absorption enhancing agent should be non-toxic and act in a reversible way on the tight junctions. Absorption enhancers that have been evaluated in recent years represent a group of compounds that differ concerning their chemical, mechanistic and toxic profiles and are broadly divided into six groups: (1) chelators, (2) surfactants, (3) bile salts, (4) fatty acids, (5) non-surfactants and (6) multifunctional polymers as summarised in table 1.1 with some examples of each group (Lee *et al.*, 1991:92).

TABLE 1.1 Classification of absorption enhancing agents with examples (Lee *et al.*, 1991:92).

CLASS	EXAMPLES
Chelating agents	EDTA, citric acid, salicylates and N-acyl derivatives of collagen
Surfactants	Sodium lauryl sulphate and polyoxyethylene-9-lauryl ether
Non-surfactants	Unsaturated cyclic ureas and 1-alkyl- and 1-alkenylazacycloalkanone derivatives
Bile salts and derivatives	Sodium deoxycholate, sodium glucocholate and sodium tauro-cholate
Fatty acids and derivatives	Oleic acid, caprylic acid, acylcarnitines, acylcholines and mono- and diglycerides
Polymers	Chitosan, TMC, carbopol and polycarbophil

Excellent reviews on the properties and effectiveness of these substances and their mechanisms of action are available in literature. (Hochman and Artursson, 1994:253; Muranishi and Yamamoto, 1994:66; Lee *et al.*, 1991:91; Lehr *et al.*, 1990:51). Only the absorption enhancing properties of the polymer chitosan and its derivative *N*-trimethyl chitosan chloride (TMC) will be discussed in further detail in this section.

1.3.1 Chitosan as absorption enhancer of hydrophilic drugs

Chitosan, a polysaccharide with structural characteristics similar to glycosamino glycan, has become of great interest as a functional material of high potential in various fields, including biomedical research. Chitosans has been used for a range of applications ranging from a food additive to a water purification agent as well as for numerous pharmaceutical applications (Aspden *et al.*, 1995:70; Hirano *et al.*, 1988:897). The potential use of chitosan as an absorption enhancer across mucosal surfaces has been well documented in recent years. However, it was Illum *et al.* (1994:1186) who first reported that chitosan is able to promote the transmucosal absorption of small polar molecules as well as peptide and protein drugs from nasal epithelia. Immediately afterwards Artursson *et al.* (1994:1358) reported that chitosan can increase the paracellular permeability of [^{14}C]-mannitol (a marker for paracellular routes) across Caco-2 intestinal epithelia. These findings attributed to chitosan polymers the property of transmucosal absorption enhancement with some favourable properties such as:

- it is not absorbed, due to its high molecular weight, and therefore not expected to display systemic toxicity,
- it intensifies the contact between the dosage form and the site of absorption due to its mucoadhesive properties, and
- it improves peptide transport across the epithelial barrier by the reversible opening of tight junctions (Lueβen *et al.*, 1994:336).

1.3.1.1 Chemical structure of chitosan

Chitin is a major structural polysaccharide found in invertebrate animals and lower plants. Chitosan [α (1→4) 2-amino-2-deoxy-β-D-glucan] with a chemical formula of $(C_6H_{11}O_4N)_n$ is obtained by the alkaline deacetylation of chitin. The chitosan molecule is a copolymer of *N*-acetyl-D-glucosamine and D-glucosamine (figure 1.5) (Paul and Sharma, 2000:5). The sugar backbone consists of β-1,4-linked D-glucosamine with a high degree of *N*-acetylation, a structure very similar to that of cellulose, except that the acetylamino group replaces the hydroxyl group on the C-2 position. If the degree of *N*-acetylation of chitin is lowered to less than 50 % it becomes soluble in acidic solutions and is referred to collectively as chitosans (Le Dung *et al.*, 1994:209). Thus, chitosan is poly(*N*-acetyl-2-amino-2-deoxy-D-glucopyranose), where the *N*-acetyl-2-amino-2-deoxy-D-glucopyranose (or Glu-NH_2) units are linked by (1→4)-β-glycosidic bonds (Ravi Kumar, 2000:1).

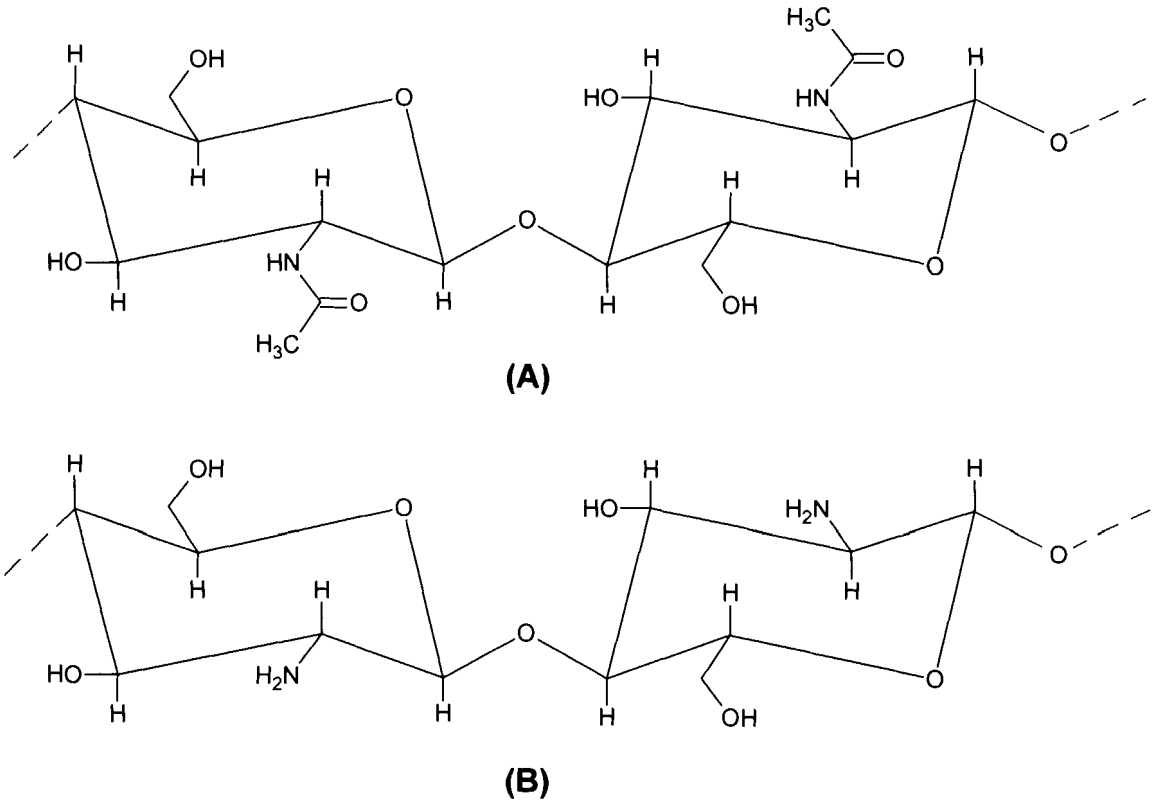


FIGURE 1.5 Structures of chitin (A) and chitosan (B) (Paul and Sharma, 2000:7).

1.3.1.2 Availability of chitosan

Chitin is the second most abundant polysaccharide in nature, cellulose being the most abundant. Chitin is easily obtained as (1) the principal component of protective cuticles of crustaceans such as crabs, shrimps, prawns, lobsters and (2) cell walls of some fungi such as *aspergillus* and *mucor* (Paul and Sharma, 1994:5). In the first case, chitin production is associated with food industries such as shrimp canning. The processing of crustacean shells mainly involves the removal of proteins and the dissolution of calcium carbonate which is present in crab shells in high concentrations. In the second case, the production of chitosan-glucan complexes is associated with fermentation processes, similar to those for the production of citric acid from *Aspergillus niger*, *Mucor rouxii* and *Streptomyces*, which involves alkali treatment yielding chitosan-glucan complexes. The alkali removes the protein and deacetylates chitin simultaneously (Ravi Kumar, 2000:2). The abundance of chitin and resulting chitosan has contributed a great deal towards the popularity of this polymer in numerous pharmaceutical applications.

1.3.1.3 Methodology for preparation of chitosan

Chitosan is marketed under a variety of forms with different molecular weights and degrees of deacetylation, or as chitosan base or salt. Shrimp or crab shells proteins are removed by boiling the shells in aqueous sodium hydroxide solution after decalcification in dilute hydrochloric acid and deproteination in a dilute sodium hydroxide solution. The sample is then deacetylated to become chitosan in a concentrated sodium hydroxide solution at boiling point. The conditions used for deacetylation will determine the polymer molecular weight and the degree of deacetylation. The purified chitosan is prepared by repeating the deacetylation process. Pharmaceutical grade chitosan is deacetylated between 90 and 95 % and the food grade between 75 and 80 % (Paul and Sharma, 2000:5).

1.3.1.4 Physicochemical and biological properties of chitosan

The word chitosan refers to a large number of polymers, which differ in their degree of *N*-deacetylation (40 - 98 %) and molecular weight (M_W) (50 000 - 2 000 000 Da) (Thanou *et al.*, 2001a:94). These two characteristics are very important to the physicochemical properties of the chitosans and hence, they have a major effect on the biological properties. Chitosan is a weak base with a pK_a value due to the D-glucosamine residue of about 5.5 to 6.5 and, therefore, is insoluble at neutral and alkaline pH values (Domard *et al.*, 1986:105). It does, however, form salts with inorganic and organic acids such as hydrochloric acid, acetic acid, glutamic acid and lactic acid. In acidic medium, the amine groups of the polymer are protonated resulting in a soluble, positively charge polysaccharide that has a high charge density (one charge for each D-glucosamine unit) (Thanou *et al.*, 2001a:94). Chitosan also has gel-forming properties in acidic medium and is used in drug delivery systems as a constituent in matrix systems and as a bioadhesive- and mucoadhesive excipient (Knapczyk, 1993:233). Chitosan is a biocompatible, slowly biodegradable and non-toxic natural polymer and has reactive hydroxyl and amino groups that can be modified chemically for various applications (Paul and Sharma, 2000:6).

1.3.1.5 Safety of chitosan as absorption enhancer

Chitosan has been used by the water purification industry for more than 20 years. When spread over the surface of water it absorbs the impurities (grease, oil, heavy metals, etc.) thus making the pollutants easy to remove (Paul and Sharma, 2000:7). In an investigation by Aspden *et al.* (1996:28) on chitosan salts of different M_W and degree of deacetylation in their ability to enhance nasal absorption of insulin, it was shown, using a rat nasal perfusion method, that chitosan salts produced minimal membrane and cellular damage when compared to laureth-9 solutions. The same authors also investigated the effect of chronic application (28 days) of chitosan to nasal epithelia of guinea pigs. Measuring the ciliary beat frequency (CBF) of excised nasal tissue, it was proven that all

chitosans tested (various degrees of deacetylation and M_W) did not influence the ciliary activity (Aspden *et al.*, 1997:137). The oral LD_{50} of chitosan in mice was further reported to be more than 16g/kg, while the US Food and Drug Administration (FDA) has approved chitosan as a food additive in animal feed (Paul and Sharma, 2000:7).

1.3.1.6 Mechanism of action of chitosan

Chitosan salts such as chitosan glutamate and chitosan hydrochloride have been shown to increase the absorption of a number of hydrophilic compounds and peptide drugs both *in vitro* and *in vivo* (Kotzé *et al.*, 1998:253). Illum *et al.* (1994:1186) showed that chitosan solutions at 0.5 % (w/v) concentration were highly effective to increase the absorption of insulin across nasal mucosa in rats and sheep. The mechanism of action of chitosan was suggested to be a combination of bioadhesion and a transient widening of the tight junctions between epithelial cells to allow for the paracellular transport of these hydrophilic molecules. Such an action is believed to be due to the interaction of the positively charged amino groups on the C-2 position of chitosan with the anionic components (sialic acid) of the glycoproteins on the surface of the epithelial cells (Schipper *et al.*, 1997:923). Furthermore, the interior of the tight junctions (pores) are highly hydrated and contain fixed negative charges. An alteration in the relative concentration of specific ion species in the pore volume would result in substantial alterations in tight junction resistance, which might lead to loosening or opening of the pores (Thanou *et al.*, 1999:74). This interaction also results in a structural reorganisation of the tight junction-associated proteins (Kotzé *et al.*, 1998:253).

1.3.1.7 Factors influencing the absorption enhancing properties of chitosan

1.3.1.7.1 Molecular weight and degree of acetylation

The molecular weight and the degree of acetylation of chitosans determine both their absorption enhancing and cytotoxic properties. Chitosans with a high degree of acetylation (35 to 49 %) increased the absorption of drugs at high molecular weights and showed low toxicity. Chitosans with lower degrees of acetylation (1 to 15 %), on the other hand, promoted drug absorption at both low and high molecular weights but also displayed clear dose dependant toxicity (Schipper *et al.*, 1997:923). Therefore, toxicity and absorption enhancement properties may be controlled by selecting a chitosan with the optimal molecular weight and degree of acetylation.

1.3.1.7.2 Charge density

Its suggested that the charge density of chitosan plays an important role in the enhancement of mucosal absorption, since the influence of this polymer on the transport of marker molecules is the strongest when the pH is well below its pK_a of 6.5 (Schipper *et al.*, 1996:1686). Chitosan is a weak base and a certain amount of acid is required to transform the glucosamine units into the positively charged, water-soluble form. Due to their charge loss in neutral and basic environments, chitosan precipitates from solution rendering it unsuitable as an absorption enhancer at physiological pH (Kotzé *et al.*, 1997b:1197) and at this pH, the molecule is most likely to exist in a coiled configuration (Artursson *et al.*, 1994:1358).

1.3.2 *N*-trimethyl chitosan chloride (TMC) as absorption enhancer of hydrophilic drugs

As previously mentioned, chitosan is effective as a penetration enhancer for hydrophilic drugs *in vivo*, when administered in an acidic environment since it is soluble at low pH values. Options for the potential use of chitosan in the more basic environment of the large and small intestines are therefore limited. In this respect, chitosan derivatives with varying physicochemical properties (e.g. water solubility at neutral and basic pH values) will be of particular interest as they might prove to be useful as absorption enhancers in these environments. Kotzé *et al.* (1999a:341) hypothesised that polymers such as unmodified chitosan with a primary amino group may not be the optimal ones, but that polymers or derivatives with different substituents, basicities or charge densities may have the same or even increased efficacy in opening tight junctions.

An ideal approach would be to modify the solubility while still retaining the biodegradability and absorption enhancing properties of chitosan. As previously mentioned, chitosan is a versatile polymer with many functional groups available for chemical modification. In the past several chitosan derivatives have been synthesised, one of which is *N*-trimethyl chitosan chloride (TMC) which has been intensely studied and described for its absorption enhancing effects (Domard *et al.*, 1986:105; Kotzé *et al.*, 1999a:341). It was concluded that the potential use of TMC, in neutral and basic environments where normal chitosan salts are ineffective as absorption enhancers, could contribute significantly to the effective delivery of hydrophilic compounds.

1.3.2.1 Chemical structure and methodology for preparation of TMC

TMC differs from chitosan in that primary amino groups on the C-2 position of chitosan, is replaced with quaternary amino groups. TMC can be synthesised by a method of reductive methylation of chitosan with methyl iodide in a strong basic environment at an elevated temperature as described by Domard *et al.*

(1986:105) and shown in figure 1.6. The degree of quaternisation can be altered by increasing the number of reaction steps or by increasing the reaction time (Sieval *et al.*, 1998:157; Hamman, 2001:76). Complete quaternisation of chitosan will probably be difficult because of the presence of acetyl groups and the potential steric effects of the attached methyl groups on adjacent quaternary amino groups (Hamman *et al.*, 2000:36).

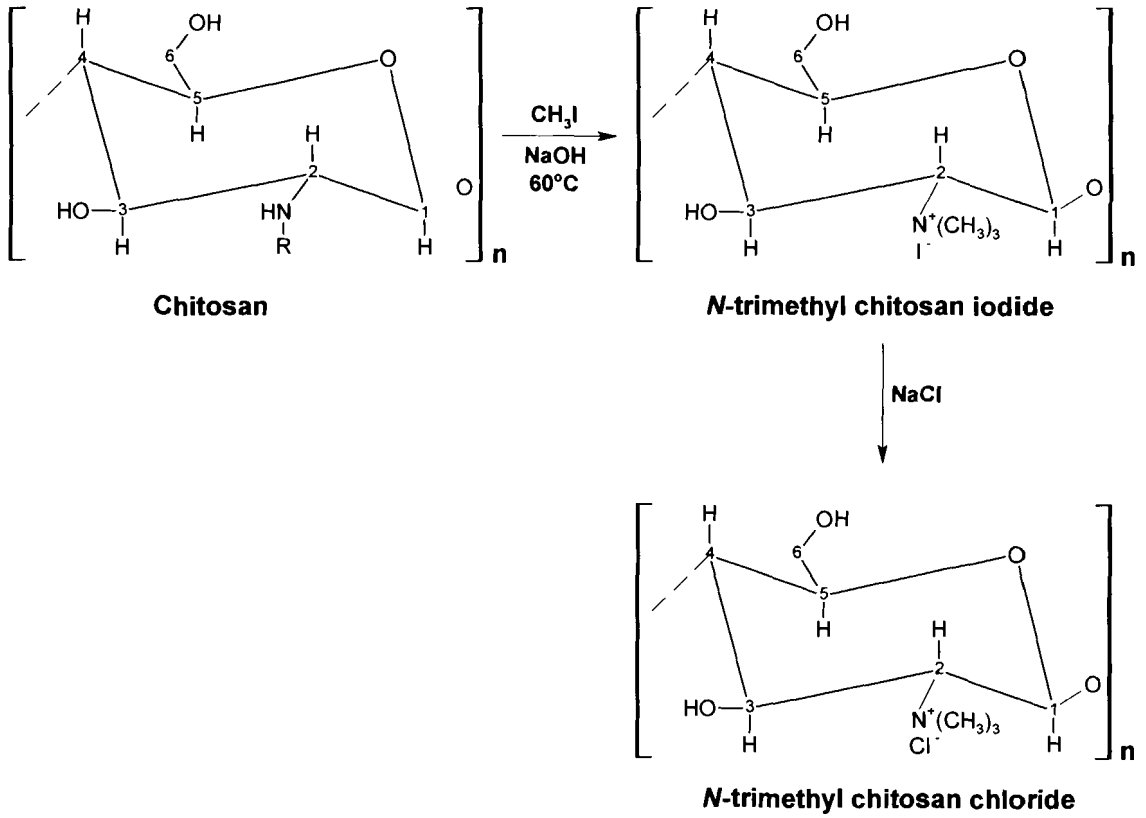


FIGURE 1.6 Synthesis and structure of *N*-trimethyl chitosan chloride (Kotzé *et al.*, 1999a:353).

1.3.2.2 Physicochemical properties of TMC

TMC is a partially quaternised derivative of chitosan with superior solubility and basicity, compared with other chitosan salts (Kotzé *et al.*, 1997b:1197). According to Kotzé *et al.* (1998:35) the initial chitosan used to synthesise TMC was only soluble in acidic solutions, but after quaternisation it became perfectly soluble in water. This increased solubility, either in basic or acidic medium, was

observed for a degree of quaternisation even as low as 10 % as determined by ^{1}H-NMR spectra. As mentioned previously, chitosan hydrochloride and chitosan glutamate are only soluble at acidic pH levels. Even at these low pH levels, it was difficult to prepare 1.5 % (w/v) solutions due to the high viscosity of the solutions. A pronounced decrease in the intrinsic viscosity of TMC, compared to the starting material, was observed.

Snyman *et al.* (2002:145) determined the absolute molecular weights, radius and polydispersity of a range of TMC polymers with different degrees of quaternisation with size exclusion chromatography (SEC) and multi-angle laser light scattering (MALLS). It was found that the absolute molecular weight of the TMC polymers decreased with an increase in the degree of quaternisation. It should be noted that the molecular weight of the polymer chain increases during the reductive methylation process due to the addition of methyl groups to the amino group of the repeating monomers. However, a net decrease in the absolute molecular weight is observed due to degradation of the polymer chain caused by exposure to the specific reaction conditions during the synthesis (Snyman *et al.*, 2002:145).

1.3.2.3 Safety of TMC as absorption enhancer

Chitosan is considered a biocompatible, biodegradable and non-toxic polymer (§ 1.3.1.5), but the properties of cationic polymers can also exhibit damaging effects on the epithelial cells as some dose dependant toxic effects for certain chitosans were reported by Schipper *et al.* (1996:1686). Therefore, for the evaluation of novel absorption enhancers, safety studies are required to guarantee the absence of tissue damaging effects of the compound under investigation. The effects of TMC on ciliary beat frequency and its possible membrane damaging effects were studied *in vitro* by Thanou *et al.* (1999:77). The effect of 1.0 % (w/v) TMC60 (degree of quaternisation 60 %) on the ciliary beat frequency of chicken embryo trachea resulted in a slight decrease in this frequency. This decrease in frequency was however less pronounced than the decrease observed after

incubation with physiological saline (0.9 % NaCl) (Thanou *et al.*, 1999:80). A fluorescent probe was used in the presence of TMC for the cytotoxic study on selected Caco-2 cell monolayers. The probe only emits fluorescence upon binding with the nuclei of cells and those cells which did not take up this fluorescent probe were considered viable. Horizontal cross-sections of cell monolayers treated with 1.0 % (w/v) TMC60 for 4 hours showed no nuclei staining when visualised with confocal laser scanning microscopy (CLSM). It was therefore concluded by the authors that TMC is a safe absorption enhancer for hydrophilic molecules across nasal and other mucosal tissues (Thanou *et al.*, 1999:81).

1.3.2.4 Mechanism of action of TMC

Schipper *et al.* (1997:923) found that the effect of chitosan on the paracellular permeability is initiated by its direct and specific binding at the cell membrane. This binding could be inhibited by heparin, indicating that the positive charge is important for the binding properties of chitosan. TMC, at all degrees of quaternisation, bears positive charges, independently of the environmental pH. It can therefore be speculated that TMC is triggering the opening of the tight junctions by a similar action on the junctional complex as chitosan (Thanou *et al.*, 2000a:23; Kotzé *et al.*, 1997a:251). Thanou (2000c:91) used CLSM to visualise the tight junctions' membrane protein, occluding, by immunocytochemistry staining in the presence and absence of TMC60 (degree of quaternisation 60 %). The effects of TMC60 on cytoskeletal F-actin were also determined by visualisation using CLSM. The transmembrane protein, occluding, displayed a disrupted pattern after incubation with 1.0 % (w/v) TMC60, suggesting that the interaction of TMC60 with the tight junctions' proteins is the major mechanism for opening the tight junctions and subsequently increased paracellular permeability (Thanou, 2000c:91).

1.3.2.5 Effect of the degree of quaternisation on the absorption enhancing properties of TMC

Kotzé *et al.* (1998:41) studied the effect of TMC (degree of quaternisation 12.28 %), chitosan hydrochloride and chitosan glutamate on the permeability of the hydrophilic marker [^{14}C]-mannitol in Caco-2 cells at a pH of 6.2. From these results, it was evident that TMC was not as effective as an absorption enhancer, at similar weight concentrations, as chitosan hydrochloride and chitosan glutamate. Its lesser efficacy was attributed to the degree of quaternisation of TMC, which determines the amount and density of the positive charges on the C-2 position of the polymer, and by a partial concealment of the positive charge on the amino group by the attached methyl groups. The amount of positive charges on TMC influences the amount of interactions with the negative sites on the cell membranes and the opening of the tight junctions.

Jonker *et al.* (2002:205) used both an *in situ* (single pass perfusion) and an *in vitro* (everted intestinal sac) method in rats to study the effect of the degree of quaternisation on the transport of [^{14}C]-mannitol. TMC polymers with a degree of quaternisation ranging from 22 to 48 % were used in these studies. It was clearly demonstrated that TMC enhances intestinal permeation in a neutral pH environment and that the extent of absorption enhancement was dependant on the degree of quaternisation of TMC. In both models the best permeation enhancing results were obtained with the highest degree of quaternisation. In general it was proposed that TMC with higher degrees of quaternisation might be more effective as absorption enhancers for the increased paracellular transport of hydrophilic compounds in neutral environments (Jonker *et al.*, 2002:205; Kotzé *et al.*, 1999b:274).

1.3.2.6 Effect of TMC on the transepithelial electrical resistance (TEER) of human intestinal epithelial cells (Caco-2)

Kotzé *et al.* (1997b:1199) proved that TMC was able to decrease the TEER of Caco-2 cell monolayers in concentrations above 1.0 % (w/v). The effect of TMC

on the TEER of Caco-2 cell monolayers as a function of time is shown in figure 1.7. Incubation with TMC (1.5, 2.0 and 2.5 % w/v) resulted in a pronounced and immediate reduction (9 ± 4, 52 ± 3 and 79 ± 0.3 % respectively, after a 20 minutes incubation period) in TEER values as a function of the concentration, compared to the control group (Kotzé *et al.*, 1997b:1199). Unfortunately this publication does not mention the pH at which the experiments were performed.

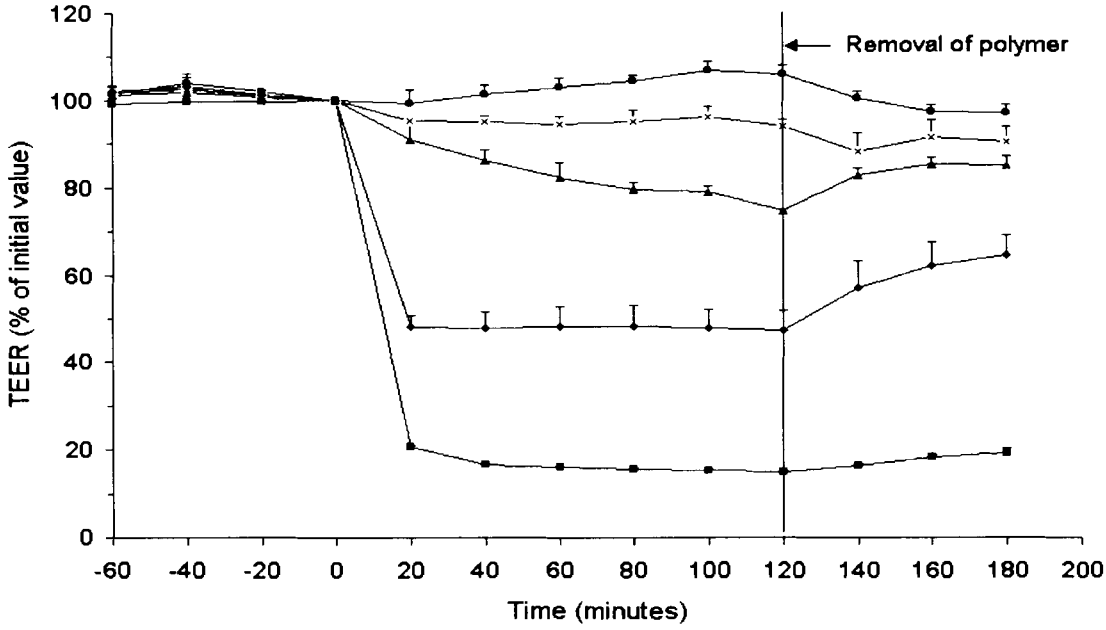


FIGURE 1.7 Effect of TMC on the TEER of Caco-2 cell monolayers. Each point represents the mean ± S.D. of 3 experiments. Keys: Control (x), TMC 1.0 % (●), TMC 1.5 % (▲), TMC 2.0 % (♦), TMC 2.5 % (■) w/v, vertical line represents start of reversibility experiment (Kotzé *et al.*, 1997b:1200).

The absence of intracellular trypan blue, a staining marker to identify damaged cells, after prolonged incubation with TMC implies that the Caco-2 cells remained undamaged and functionally intact. Reversibility of the effect towards the initial value can be seen from figure 1.7, especially at 1.5 and 2.0 % (w/v) concentrations of TMC. With removal of the polymer solutions, repeated washing and substituting the apical medium again with fresh Dulbecco's Modified Eagle Medium (DMEM), monolayers started to recover slowly and a slight

increase in transepithelial electrical resistance towards the initial values were found. Complete removal of the polymers, without damaging the cells proved to be difficult due to the high viscosity of the solutions and this may be the reason why the increase in resistance were only gradual (Kotzé *et al.*, 1997b:1199).

1.3.2.7 Effect of TMC on the absorption enhancement of hydrophilic model compounds

Substances such as [^{14}C]-mannitol and [^{14}C]-polyethylene glycol (PEG-4000) are metabolically inert and are highly hydrophilic in nature. Mannitol has previously been used to follow changes in the epithelial integrity of mucosal cells. Both compounds show little diffusion into the cell membranes, but are transported through the alternative aqueous paracellular pathway (i.e. through the tight junctions) and are therefore ideal substances to detect changes in the permeability when paracellular absorption enhancement studies are performed (Artursson *et al.*, 1994:1358; Borchard *et al.*, 1996:131).

Lueβen *et al.* (1996:1668) proved that the intraduodenal administration of buserelin with chitosan hydrochloride in a gel formulation increased the absolute bioavailability of buserelin from 0.1 to 5.1 %. In another study, the nasal application of insulin with chitosan glutamate led to a significant reduction in blood glucose levels in rats and sheep (Illum *et al.*, 1994:1186). Kotzé *et al.* (1997b:1197) also showed that TMC, with a degree of quaternisation of 12 %, was able to increase the transport of fluorescein isothiocyanate-labelled dextran (FD-4) across Caco-2 cell monolayers. The transport of this large hydrophilic model compound (M_W = 4400 Da) was increased 167-, 274- and 373-fold with 1.5, 2.0 and 2.5 % (w/v) concentrations of TMC, respectively.

1.4 IMPORTANCE OF MUCO/BIOADHESION

Bioadhesion can be described as the attachment of a synthetic or biological macromolecule to a biological tissue. An adhesive bond may form with either the

epithelial cell layer, the continuous mucus layer or a combination of the two. The term mucoadhesion is used specifically when the bond involves mucus coating and an adhesive polymeric device (Vasir *et al.*, 2003:14). Incorporation of bioadhesive polymers in a formulation has been considered as a useful method to localise the dosage form at a specific site and to prolong and control drug release from the delivery system. Mucoadhesive systems are designed to adhere to the mucosal surface of biological tissues and offer various advantages such as:

- longer residence time of the dosage form on mucosal tissues which may improve absorption of the drug and increase the drug's bioavailability;
- higher drug concentration at the site of adhesion-absorption which may create a driving force for the paracellular passive uptake;
- immediate absorption from the bioadhesive drug delivery system without previous dilution and possible degradation in the luminal fluids;
- enhancement of topical action of certain drugs delivered to targeted regions for the treatment of specified intestinal diseases. Better stability and longer residence time may allow more of the drug to penetrate through the mucus layer to act locally (Lueβen *et al.*, 1994:330).

The high viscosity causes the mucus a potential barrier that may inhibit the diffusion of drugs (and delivery systems) and therefore it is essential to understand the structure and physical chemistry of mucus if the gastrointestinal tract is to be exploited as a site for drug delivery.

1.4.1 Physiology of mucin

The mammalian gastrointestinal tract (GIT) is lined with a continuous layer of mucus which lubricates food masses, facilitates movement of the chyme and protects the underlying tissue from gastric juice, proteases, pathogenic micro-organisms as well as mechanical trauma. In humans, the thickness of the mucus layer through the GIT has a mean of 192 μm. The gel-forming components of

the mucus are glycoproteins (mucins) of molecular weight higher than 2 x 10^6 Da (Gabor *et al.*, 2004:462). These macromolecular polymers are highly expanded random coils made up of monomeric glycoproteins and make up between 0.5 and 5.0 % of the fully hydrated mucus secretion. The subunits of the macromolecule are coupled by peptide linkages and intramolecular cysteine-cysteine disulphide bridges (Peppas and Sahlin, 1996:1553). Mucin consists mainly of carbohydrates and there are between 2 and 20 residues per side chain. These side chains contain alternating *N*-acetylglucosamine and galactose residues and have varying degrees of branching. Residues of ester sulphates appear at intermediate positions, while fructose and sialic acid occur at the terminal ends. At a pH greater than 2.6, the sialic acid and sulphate residues are fully ionised. This confers a net negative charge to the molecule. Over 50 % of the oligosaccharides contain acid groups and in response to an irritant, the amount of acidic side chains in the glycoprotein increases to 80 %, making the macromolecule even more negatively charged (Peppas and Sahlin, 1996:1554). Therefore, charge interactions may have a significant effect on the behaviour of mucus glycoproteins and its interactions with drug delivery systems.

1.4.2 Mechanisms of adhesion

Different mechanisms of adhesion have been discussed in the literature, while the mechanisms involved in the formation of a bioadhesive bond are not completely clear. Each theoretical development has distinct limitations and the operative mechanism which is observed, is probably a combination of several of them. The process involved in a bioadhesive bond between polymer hydrogels and mucosal tissue include the following: (1) wetting and swelling of the polymer to permit intimate contact with biological tissue, (2) interpenetration of bioadhesive polymer chains and entanglement of polymer and mucus chains, and (3) formation of weak chemical bonds between entangled chains (Chickering and Mathiowitz, 1999:8). From a molecular point of view, several mechanisms have been proposed to explain the interaction of a bioadhesive polymer and a

biological surface, such as mucus, in order to create a mucoadhesive bond. The following are possible mechanisms to explain mucoadhesion (Vasir *et al.*, 2003:14):

- The *Electronic theory* suggests that electron transfer upon contact of the polymer with the mucus glycoprotein network between the two electronic structures of the surfaces can contribute to the formation of a double layer of electrical charge at the mucoadhesive interface.
- The *Adsorption theory* analyses the phenomenon in terms of the forces manifesting themselves during mucoadhesion. The adsorption theory states that the bioadhesive bond formed is due to van der Waals interactions, hydrogen bonds and related forces.
- The *Fracture theory* examines the force necessary to separate the two surfaces after the mucoadhesive bond has been established.
- The *Wetting theory* is based on the ability of mucoadhesive polymers to spread onto and develop intimate contact with the mucus surface. Thus expressions of the interfacial tension are obtained which can be used to screen various polymers for their ability to adhere to tissues.
- The *Diffusion* or *Interpenetration theory*, which has the interpenetration of macromolecular chains at the polymer-polymer interface as its basis. The bond strength increases with the degree of penetration of the polymer chains into the mucus.

1.4.3 Mucoadhesive properties of chitosan and TMC

1.4.3.1 Chitosan

The mucoadhesive properties of chitosan were described by Lehr *et al.* (1992:43). This property is probably mediated by ionic interactions between the positively charged amino groups of chitosan and the negatively charged sialic acid residues of mucus glycoproteins (Lehr *et al.*, 1992:43) and may be attributed to the following polymer characteristics:

- strong hydrogen bonding groups like -OH and –COOH,
- strong charges,
- high molecular weight,
- sufficient chain flexibility, and
- surface energy properties favouring spreading into mucus (Peppas and Buri, 1985:258).

Lehr *et al.* (1992:43) demonstrated that chitosan in the swollen state, is an excellent mucoadhesive on porcine intestinal mucosa and is also suitable for repeated adhesion. In the same study polycarbophil, another high molecular polymer, failed the repeated adhesion test. It was also shown that molecular weight played a significant role in the mucoadhesive properties of chitosan with the best results obtained at higher molecular weights (Lehr *et al.*, 1992:46). The authors also reported that chitosan underwent minimal swelling in artificial intestinal fluid due to its poor aqueous solubility at neutral pH values, proposing that the swelling could be improved by substitution of the free amino groups with short alkyl chains. This process will cause an increase in the pK_a and hence the ionisation of these groups at a higher pH, thus possibly also increasing mucoadhesion.

1.4.3.2 *N*-trimethyl chitosan chloride

Snyman *et al.* (2003:59) investigated the mucoadhesive properties of TMC polymers with different degrees of quaternisation (varying between 22 and 49 %). TMC was found to have a lower intrinsic mucoadhesivity if compared to the chitosan salts, chitosan hydrochloride and chitosan glutamate, but if compared to the reference polymer, pectin, TMC possesses superior mucoadhesive properties. The decrease in the mucoadhesion of TMC compared to the chitosan salts was attributed to a change in the conformation of the TMC polymers due to interactions between the fixed positive charges on the quaternary amino groups, which possibly also decreased the flexibility of the polymer molecules. The

interpenetration into the mucus layer by the polymer is influenced by a decrease in flexibility resulting in a subsequent decrease in mucoadhesivity.

1.5 CONCLUSION

It is evident from chapter 1 that the human gastrointestinal tract functions as a system for storage, digestion and absorption of different compounds and possesses unique characteristics along its length. The stomach functions mainly as a processor organ and displays very hostile (acidity, short resident time etc.) conditions towards its contents. The small instestine on the other hand has a very large surface area which aids in absorption of digested compounds and might display a more acceptable environment for dosage form delivery.

Polymers with a large number of derivatisable groups, a wide range in molecular weight, varying chemical composition and being stable, safe and biodegradable, offers properties preferable for the development of conventional and novel pharmaceutical products. Chitosan is an abundant natural and biocompatible polymer, obtained by alkaline *N*-deacetylation of chitin. When in solution (protonated), chitosan is able to interact with the tight junctions and provoke their opening allowing for paracellular permeation of hydrophilic drugs. Chitosan's effects on the integrity of the epithelium or the cell membranes are minimal when compared to the effects of known absorption enhancers. The problem of chitosans' ineffectiveness at neutral pH values can be surmounted by derivatisation at the amine group that renders the polymer soluble.

The partially quaternised derivative of chitosan, TMC, shows excellent solubility over a wide pH range, suggesting that it can be used as an absorption enhancer in neutral and basic environments. These enhancing effects are demonstrated by a decrease in TEER values across epithelial cell monolayers (Caco-2), as well as the observed increase in transport of hydrophilic compounds across these monolayers at neutral pH values. It has also been shown that the degree of quaternisation of TMC plays an important role in its absorption enhancing

properties, especially in neutral environments. TMC possesses mucoadhesive properties and showed no indication of epithelial damage or cytotoxicity, whereas its effect on the tight junction's integrity appears to be reversible.

It is therefore expected that the inclusion of chitosan and/or TMC into a suitable peroral drug delivery system might enhance the bioavailibility of hydrophilic drugs, provided that sufficient quantities of these polymers are included into such a system and is subsequently released at the site of drug absorption. In studies performed in the past, TMC has either been administered as a solution, or the quantities released from solid oral dosage forms were found to be inadequate for absorption enhancement of hydrophilic compounds. In the next chapter the properties of a possible drug delivery system for the release of TMC and a model hydrophilic drug will be discussed.

CHAPTER 2

CHITOSAN MICROSPHERES AS DRUG DELIVERY DEVICES

The last two decades in the pharmaceutical industry have witnessed a leading interaction among the fields of polymer and material science, resulting in the development of novel drug delivery systems. Carrier technology offers an intelligent approach for drug delivery by coupling the drug to a carrier particle such as microspheres, nanoparticles, liposomes, etc. which modulates the release and absorption characteristics of the drug. Microspheres constitute an important part of these particulate drug delivery systems (DDS) by virtue of their small size and efficient carrier characteristics. However, the success of these novel DDS can be limited due to a potential short residence time at the site of absorption. It would, therefore, be advantageous to have a means for providing an intimate contact of the DDS with the absorbing membranes. This can be achieved by coupling bioadhesion characteristics to microspheres and developing novel delivery systems referred to as 'bioadhesive microspheres' (Mathiowitz *et al.*, 1999:9).

Microspheres, in general, have the potential to be used for targeted and controlled drug delivery; but coupling of bioadhesive properties to microspheres may have additional advantages, e.g. efficient absorption and enhanced bioavailability of the drugs due to a high surface to volume ratio and a much more intimate contact with the mucus layer. Microspheres bioengineerd with bioadhesive and biodegradable polymers, keep the drugs in close proximity to their absorption window in the GI mucosa and therefore improve the absorption and oral bioavailability of drugs. Bioadhesive microspheres include microparticles and microcapsules or -beads (having a core of the drug) of 1 to 1000 µm in diameter. These microspheres may consist either entirely of a bioadhesive polymer or may only have an outer coating of it (Mathiowitz *et al.*,

1999:20). Microspheres with an average size diameter of 1000 µm will further collectively be referred to as microbeads. In this chapter, the synthesis and uses of chitosan microbeads will be discussed, as well as a brief summary on the physico-chemical properties of ibuprofen as model compound for inclusion in such microbeads.

2.1 CHITOSAN MICROBEADS

Chitosan is a non-toxic and biocompatible polymer with bioadhesive properties, which increases retention at the site of application (see chapter 1). Chitosan is a polycationic polymer, well known for its chelating properties (Berger *et al.*, 2004:27). Therefore, reactions with negatively charged components, either ions or molecules, can lead to the formation of a network through ionic bridges between polymeric chains. Ionic interactions between the negative charges of the crosslinker and the positively charged groups of chitosan are the main interactions inside such a network (Berger *et al.*, 2004:27). The use of chitosan microbead-based therapy allows drug release to be carefully tailored to the specific treatment site through the choice and formulation of various drug-polymer combinations. Using innovative microencapsulation technologies, and by varying the copolymer ratio and the molecular weight of the polymer, etc., chitosan microbeads can be developed into an optimal drug delivery system for a particular drug. Being small in size, microbeads also have large surface to volume ratios, increasing the effective surface area of the drug incorporated and enhancing the dissolution rate of the drug from the delivery system (Sinha *et al.*, 2004:3).

2.2 PREPARATION OF CHITOSAN MICROBEADS

Reacting chitosan with controlled amounts of multivalent anion results in crosslinking between the chitosan molecules. The crosslinking may be achieved

in acidic, neutral or basic environments depending on the method applied. A wide range of techniques for the preparation of chitosan microbeads are described in the literature and only the main processes will be discussed.

2.2.1 Solvent evaporation method

This method is the most commonly used for microencapsulation and involves the formation of an emulsion between a polymer solution and an immiscible continuous phase, whether aqueous (o/w) or non-aqueous (w/o) (Sinha *et al.*, 2004:7). A buffered or non-buffered aqueous solution of the drug is added to an organic phase consisting of the polymer solution in solvents like dichloromethane (or ethyl acetate or chloroform) with vigorous stirring to form the primary water in oil emulsion. This emulsion is then added to a large volume of water containing an emulsifier to form the multiple emulsion (w/o/w). The multiple emulsion is then subjected to stirring until most of the organic solvent evaporates, leaving solid microbeads. The beads can then be washed, centrifuged and lyophilised to obtain the free flowing and dried product (Vasir *et al.*, 2003:19).

2.2.2 Spray-drying

Chitosan microbeads can also be prepared by spray-drying. In this process, the drug may be dissolved or dispersed in the chitosan solution, which is sprayed, air-dried and then followed by the addition of a crosslinking agent (Sinha *et al.*, 2004:7). The size of the beads can be controlled by the rate of spraying, the feeding rate of polymer-drug solution, nozzle size and the drying temperature. This method of microencapsulation is particularly less dependant on the solubility characteristics of the drug and polymer, and is simple and reproducible (Vasir *et al.*, 2003:20).

2.2.3 Hot melt microencapsulation

Using this method, the chitosan is first melted and then mixed with solid particles of the drug that have been sieved to less than 50 µm. The mixture is suspended in a non-miscible solvent (e.g. silicone oil) through continuous stirring, and then heated to 5 °C above the melting point of the polymer. Once the emulsion is stabilised, it is cooled until the polymer particles solidify. The resulting microsbeads are washed by decantation with petroleum ether. The size distribution of the beads can be controlled by altering the stirring rate. The only disadvantage of this method is the moderate temperature to which the drug is exposed (Vasir *et al.*, 2003:20).

2.2.4 Crosslinking with other chemicals

Crosslinking agents such as gluteraldehyde, formaldehyde and genipin have been used for preparation of chitosan microbeads (Sinha *et al.*, 2004:6). Ngah *et al.* (2002:182) sprayed a solution of chitosan in acetic acid into a precipitation bath containing 0.5 M sodium hydroxide solution. The acetic acid neutralised the chitosan gel and thereby coagulated the chitosan gel to spherical uniform chitosan beads. The wet beads were then suspended in a gluteraldehyde solution (chitosan/gluteraldehyde ratio of 1:1) for 24 hours. After crosslinking, the crosslinked chitosan were insoluble in acidic and alkaline medium. The swelling of the crosslinked chitosan beads were also reduced significantly (Ngah *et al.*, 2002:181). It is therefore evident that the chemical stability of the chitosan is greatly improved by crosslinking, which is not necessarily ideal for drug delivery.

2.2.5 Ionotropic gelation

The principle on which ionotropic gelation is based depends on the attraction between a positive and a negative charge. Ionically crosslinked chitosan networks can be divided into two groups, depending on the type of crosslinker,

either anions or anionic molecules, used. However, most of their characteristics and properties are identical (Berger *et al.*, 2004:27). The counterions used for ionotropic gelation can be divided into three categories: (1) low molecular weight counterions (e.g. tripolyphosphate), (2) hydrophobic counterions (e.g. alginate) and (3) high molecular weight ions (e.g. lauryl sulphate). Their nature depends on the type of crosslinker used (Sinha *et al.*, 2004:3). In order to prepare a polymeric network containing ionically crosslinked chitosan, one needs at least a charged ionic crosslinker and chitosan dispersed in a solvent, commonly water.

Microbeads made of chitosan are produced by dissolving the polymer in an aqueous solution, suspending the active ingredient in the mixture and extruding through a precision device, producing microdroplets which fall into a hardening bath, which is slowly stirred. The hardening bath usually contains a pentasodium tripolyphosphate (TPP) or sodium sulphate solution. A network is formed in the presence of the negatively charged entities, which form bridges between the positively charged chitosan polymeric chains (Vasir *et al.*, 2003:20). A bead is formed on contact with the solution within the hardening bath, entrapping the drug within the three-dimensional network of ionically linked polymer. The microbeads are separated after a proper stirring time and briefly rinsed with deionised water to remove the excess crosslinker solution from the surface of the beads. The porosity of the beads depends on the method of drying. Freeze-drying results in more porous beads than oven-drying. The particle size of microbeads can be controlled by using various size extruders or by varying the polymer solution flow rate. This method of preparing chitosan microbeads is well established and easy to perform (Bodmeier *et al.*, 1989:1480).

2.3 FACTORS AFFECTING THE ENTRAPMENT EFFICIENCY OF DRUGS IN CHITOSAN MICROBEADS

Many factors affect the entrapment efficiency of the drug in the chitosan microbeads, e.g. nature of the drug, chitosan concentration, drug/polymer ratio,

stirring speed during synthesis, etc. A number of reports have shown that entrapment efficiency increases with an increase in chitosan concentration. This may be explained on the basis that an increase in viscosity of the chitosan solution with increase in concentration prevents drug crystals from leaving the droplet. Generally a low concentration of chitosan shows low encapsulation efficiency. However, at higher concentrations, chitosan forms highly viscous solutions, which are difficult to process (Sinha *et al.*, 2004:8). Genta *et al.* (1998:779) obtained satisfactory ketoprofen contents in all batches of chitosan microspheres with a theoretical polymer/drug ratio of 1:2 (w/w). Microspheres made with a mixture of high M_W/low M_W chitosan (1:2 w/w) showed good drug content and encapsulation efficiency and these were independent of polymer/drug ratio. Maximum drug encapsulation efficiency was obtained for the lowest theoretical drug/chitosan ratio (Genta *et al.*, 1998:779). Other factors that might influence the drug loading of beads are:

- the solubility of the drug in the chitosan solution,
- the solubility of the drug in the external phase or gelling agent,
- the pK_a value of the drug and the pH of the chitosan solution and the external phase,
- the method of drying the beads, and
- the volume of the external phase (Shu and Zhu, 2001:218).

2.4 PARAMETERS AFFECTING THE RELEASE CHARACTERISTICS OF DRUGS FROM CHITOSAN MICROBEADS

Many parameters determine the drug release behaviour from chitosan microbeads. These include concentration and molecular weight of the chitosan, the type and concentration of crosslinking agent, variables like stirring speed, additives, drug to chitosan ratio, etc. (Sinha *et al.*, 2004:10). A few of these factors will be discussed in more detail.

2.4.1 Effect of molecular weight (M_W) of chitosan

Drug release studies from chitosan beads have generally shown that the release of the drug decreased with an increase in molecular weight of chitosan (Sinha *et al.*, 2004:10). Polk *et al.* (1994:178) reported that chitosan M_W was a key variable in the release of albumin from chitosan microspheres. The M_W of chitosan was varied from 1.25×10^6 to 0.25×10^6 through a nitrite oxidation reaction with sodium nitrite. Decreasing the M_W increased the release of albumin. In another study, Shiraishi *et al.* (1993:217) reported the release rate of indomethacin from chitosan hydrolysate gel beads to decrease with increasing molecular weight of chitosan. However, Genta *et al.* (1998:779) reported that the fastest ketoprofen dissolution profile from chitosan microspheres was obtained from medium M_W chitosan. This may be attributed to the swelling behaviour of chitosan micropsheres. An increase in the molecular weight of chitosan leads to an increase in viscosity of the gel layer, which influences the diffusion of the drug as well as erosion of the microspheres.

2.4.2 Effect of concentration of chitosan

Aiedeh *et al.* (1997:567) studied a method of chitosan interfacial crosslinkage by ascorbyl palmitate in water/oil dispersion. The microspheres obtained had release kinetics approaching zero order and a release rate which could be increased by decreasing the chitosan content in the preparative solution. The rate of cisplatin release also reduced with the increasing concentration of chitosan (Sinha *et al.*, 2004:10). Thus, the rate of drug release from chitosan microbeads might be modified by altering the chitosan concentration in the polymer solution.

2.4.3 Effect of drug content

A number of reports studying the effect of drug release have shown that the release of the drug from the microspheres increases with increase in drug content in the microspheres (Sinha *et al.*, 2004:11). However, contrary results have also been reported. Bodmeier *et al.* (1989:1475) found that the release of sulfadiazine (a water insoluble drug) decreased with increase in drug content in the chitosan microspheres. The effect of the drug content inside the microbeads on the release characteristics is therefore, not completely clear and might be influenced by other factors e.g. the physico-chemical properties of the incorporated drug.

2.4.4 Physical state of the drug

The physical state of a drug is also an important parameter influencing drug release characteristics while investigating the drug release kinetics from a dosage form and may vary from molecular dispersion to well defined crystalline structures. It was shown that cimetidine and famotidine were released with a burst effect when molecularly dispersed in the form of a solid solution inside the microspheres (He *et al.*, 1999:53). Drug release might therefore decrease when the physical state of the drug tends to be more crystalline inside the beads, due to the drug crystals being entrapped inside the crosslinked polymer network.

2.4.5 Effect of density of crosslinking

The crosslinking density has a remarkable effect on the release of drugs from the microspheres. Jameela *et al.* (1998:17) reported that lightly crosslinked microspheres released 70 % of progesterone in 40 days compared to only 35 % release from highly crosslinked microspheres. In a study by Ko *et al.* (2002:165), felodipine loaded chitosan microparticles were prepared by ionic crosslinking with tripolyphosphate (TPP). On examining the morphologies of chitosan-TPP

microparticles with scanning electron microscopy (SEM), it was observed that microparticles showed more spherical shapes and smooth surfaces when the pH of TPP solution is decreased and the M_W of chitosan is increased. Chitosan microparticles prepared at a lower pH or higher concentrations of TPP solutions resulted in slower felodipine release. When the molecular weight and concentrations of chitosan solutions are decreased, release of the drug was found to increase. The release of the drug from chitosan-TPP microparticles was decreased when crosslinking time was increased (Ko *et al.*, 2002:165).

In a study of glutaraldehyde crosslinked chitosan microspheres containing the hydrophilic drug hydroquinone, it was found that slower drug release rates were obtained from microspheres prepared by using a higher initial concentration of chitosan, a higher molecular weight of chitosan and/or a lower drug concentration. It was established that the release rate of hydroquinone was mainly controlled by the polymer crosslinking density and the degree of swelling of the hydrogel matrix (Dini *et al.*, 2003:375). Therefore, increasing the polymer crosslinking density might result in a decrease in drug release from chitosan microbeads.

2.5 STABILITY OF CHITOSAN MICROBEADS

A few studies have reported the instability of chitosan microspheres (prepared by precipitation) in acidic medium (Sinha *et al.*, 2004:12). The acid instability of the microspheres can be explained by the manufacturing process of the microspheres that were used. The addition of sodium sulphate to an acetic acid solution of chitosan leads to poorly soluble chitosan derivatives by ionic neutralisation of the positively charged amino groups. On addition of the acid (increased proton concentration) the equilibrium is shifted towards solubilisation of chitosan and the microspheres dissolve (Illum, 1998:1329). Bodmeier *et al.* (1989:1475) prepared sulfadiazine loaded chitosan beads using tripolyphosphate and observed that the beads had poor mechanical strength. This might be

attributed to a low TPP concentration and short stirring time, resulting in poor crosslinking.

2.6 PHARMACEUTICAL APPLICATIONS OF DRUG LOADED CHITOSAN MICROBEADS

2.6.1 Controlled drug release

The idea of controlled release from polymers dates back to the 1960s through the employment of silicone rubber and polyethylene. The lack of degradability in these systems implied the requirement of eventual surgical removal and thus limited their applicability. In the 1970s biodegradable polymers were suggested as appropriate drug delivery materials circumventing the requirement of removal. The idea of polymer microcapsules as delivery systems was reported as early as the 1960s and degradation was incorporated through the employment of a degradable polymer coating (Freiberg and Zhu, 2004:1). The development of peroral controlled release drug delivery systems has been hindered by the inability to restrain and localise the delivery system in selected regions of the gastrointestinal tract (GIT) and therefore, bioadhesive drug delivery systems form an important approach to decrease the GI transit of drugs.

Diclofenac sodium (DFS) is a potent non-steroidal anti-inflammatory drug with pronounced analgesic and antipyretic properties. Due to DFS's short half life (1-2 hours) and its gastrointestinal adverse effects such as bleeding, ulcerations or perforation of the intestinal walls, it would be preferable to administer DFS in a controlled drug release device to lengthen the therapeutic effect and to decrease the side effects (Gupta and Ravi Kumar, 2000:1116). Drug release studies, conducted on DFS loaded chitosan beads by Gupta and Ravi Kumar (2000:1116), displayed a higher drug bioavailability from chitosan beads in a medium with an acidic pH, than in a medium with a basic pH.

2.6.2 Targeted drug delivery

2.6.2.1 Colon specific drug delivery

Drug targeting to the colon for topical or systemic delivery is very useful in the treatment of various diseases of the colon, which include ulcerative colitis, Chron's disease and colon carcinomas. In general, four primary approaches have been proposed for targeted colon delivery of peptides and other labile drugs, namely:

- prodrugs,
- pH-dependant systems,
- time-dependant systems, and
- microflora-activated systems (Yang *et al.*, 2002:2).

The triggering mechanism for drug release with *prodrugs* is based on cleavage of the linkage bond between the drug and the carrier via reduction and hydrolysis by enzymes (e.g. azoreductase, glycosidase and glucuronidase) from colon bacteria. The prodrug is able to achieve site specificity, although it will be considered as a new chemical entity from a regulatory perspective. So far, this approach has been primarily confined to actives related to the treatment of inflammatory bowel disease (IBD) including Crohn's disease and ulcerative colitis (Yang *et al.*, 2002:2).

pH dependant drug release utilises a combination of polymers with pH-dependant solubilities to take advantage of the pH changes along the GI tract. This design exhibits unpredictable site-specificity of drug release due to inter-/intra patient variation as well as the similarity in pH between the small intestine and the colon (Yang *et al.*, 2002:2).

The onset of drug release from *time-dependant systems* is aligned with positioning the delivery system in the colon by incorporating a time factor simulating the environment in the upper GI tract. Even though the transit times in the small intestine are rather consistent, high variation of gastric retention times

makes this approach complicated in predicting the accurate location of drug release (Yang *et al.*, 2002:2).

Microflora-activated systems primarily use the fermentation of non-starch polysaccharides by colon anaerobic bacteria to trigger drug release. The polysaccharides are incorporated into the delivery system via film-coating and matrix formation. Various pectinolitic enzymes which are responsible for the degradation of pectin are present in the colon, making pectin an ideal candidate for colon specific drug delivery. Drug containing pectin beads can be prepared by ionotropic gelation which undergoes enzymatic degradation after arrival in the colon, resulting in drug release (Aydin and Akbuğa, 1996:133). This colon specific drug delivery strategy is highly promising because non-starch polysaccharides can only be degraded in the colon. However, it should be pointed out that enzymatic degradation of a polysaccharide matrix is a slow process, usually requiring over 12 hours for complete degradation (Yang *et al.*, 2002:2).

2.6.2.2 Floating alginate beads for stomach specific drug release

Intragastric floating dosage forms are useful for the administration of drugs that have a specific absorption site, are insoluble in the intestinal fluid, or are used for the treatment of gastric diseases (e.g. peptic ulceration). An important factor influencing the design of stomach specific dosage forms is the gastric emptying time (GET). Prolongation of the GET is one of the main goals in the controlled delivery of such a device (Murata *et al.*, 2000:221). Various advantages exist for the development of stomach specific drug delivery. Floating systems have a density lower than the density of the gastric juice and therefore the gastric residence time, and hence the bioavailability of drugs that are absorbed in the upper GI tract, will be improved. Chitosan beads having internal cavities with air entrapped within a multiple-unit compartment system results in excellent buoyancy. This gastrointestinal transit-controlled preparation is designed to float on the surface of gastric juice, prolonging the gastric retention time (GRT) of the

system (Sato *et al.*, 2004:98). Murata *et al.* (2000:221) prepared floating alginate beads by incorporating vegetable oil or chitosan flakes into them. Beads prepared in this manner maintained metronidazole serum concentrations of up to 4 hours. Sufficient amounts of drug also reached the gastric mucosa making it a controlled delivery device as well as stomach specific (Murata *et al.*, 2000:221).

2.6.3 Protein and peptide drug delivery

Protein and peptide drugs offer formidable challenges for peroral delivery due to their relatively large size, enzymatic degradation and very low permeability across the absorptive epithelial cells. Bioadhesive microspheres provide an interesting non-invasive patient compliant approach to improve the absorption of these drugs. The luminal enzymatic degradation of proteins and peptides can be effectively minimised by direct contact with the absorptive mucosa and avoiding exposition to body fluids and enzymes. Specific enzyme inhibitors can also be attached to the surface of bioadhesive microspheres (Vasir *et al.*, 2003:26). Moreover, chitosan have been reported to possess permeability enhancing properties (see § 1.3.1) and may further enhance the oral bioavailability of the high molecular weight and hydrophilic protein and peptide drugs (Kotzé *et al.*, 1997a:244).

2.7 IBUPROFEN AS MODEL DRUG

Arylpropionic acid derivatives represent a group of effective, useful non-steroidal anti-inflammatory drugs (NSAIDs). They may offer significant advantages over aspirin and indomethacin for many patients, since they usually are better tolerated. Nevertheless, propionic acid derivatives share all of the detrimental features of the entire class of drugs (Jackson Roberts II and Morrow, 2001:710). Ibuprofen was introduced in the late sixties as a safe NSAID for the treatment of a wide range of indications, including pain, inflammation, arthritis, fever and dysmenorrhea.

2.7.1 Identification

2.7.1.1 Chemical denominations

Ibuprofen is a non-steroidal anti-inflammatory drug and belongs to the propionic acid derivatives of NSAIDs. The compound is also known as (±)-2-(4-*iso*butylphenyl) propionic acid, (±)-(α-methyl-4(2-methyl-propyl) benzeneacetic acid and p-isobutyl-hydratropic acid (USP, 2004:953).

2.7.1.2 Structure, formula and molecular mass

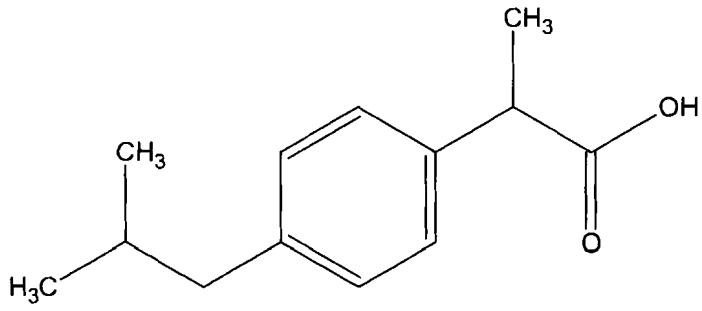


FIGURE 2.1 The chemical structure of ibuprofen (USP, 2004:953).

Molecular formula: $C_{13}H_{18}O_2$ (Budavari, 2001:4906).

Molecular weight: 206.281 (USP, 2004:953).

2.7.1.3 Description

Ibuprofen occurs as a white powder or a colourless, crystalline stable solid with a melting point of 75 °C to 77 °C (Budavari, 2001:4906). Higgins *et al.* (2001:281) obtained the ultraviolet absorption spectrum of ibuprofen in methanol and 0.1 N NaOH. The spectrum consists of the weak absorption bands of the phenyl ring that are found in the 255 to 275 nm range, for which the molar absorptivity is

approximately 250 l/mol.cm. The other strong feature consists of the much more intense band system centred around 225 nm, for which the molar absorptivity is approximately 9000 l/mol.cm (Higgins *et al.*, 2001:281).

2.7.2 Physico-chemical characteristics

2.7.2.1 Solubility

The substance is readily soluble in alcohols, chlorinated hydrocarbon solvents and dimethyl sulfoxide, but is only sparingly soluble in non-polar hydrocarbon solvents. In aqueous conditions summarised in table 2.1, ibuprofen is seen to be nearly insoluble at low pH, but readily soluble in basic media. The solubility of ibuprofen is also affected by stereochemistry. At pH 1.5 ibuprofen has an aqueous solubility of 4.6 mg/100 ml, whereas the enantiomere R(-) and S(+) forms have solubilities of 9.6 mg/100 ml and 9.5 mg/100 ml, respectively (Higgins *et al.*, 2001:277).

TABLE 2.1 Solubility of ibuprofen in aqueous systems at 20 °C (Higgins *et al.*, 2001:278).

SYSTEM COMPOSITION	SOLUBILITY (MG/ML)
Deionised water (pH 3.0)	< 0.1
HCl (pH 1.0)	< 0.1
Phosphate buffer (pH 4.0)	< 0.1
Phosphate buffer (pH 6.0)	1.0
Phosphate buffer (pH 8.0)	> 100.0

2.7.2.2 Polymorphism

Other than its tendency to adopt a variety of particle morphologies, ibuprofen does not appear to exhibit genuine polymorphism. Additionally, although polymorphs of ibuprofen have not been reported, the lysine salt of ibuprofen has been shown to exist in two polymorphic forms. It should be noted that ibuprofen materials manufactured by different sources can have different crystal dislocation, surface rugosity and/or surface areas (Higgins *et al.*, 2001:272).

2.7.3 Stability

In the solid-state, ibuprofen is considerably stable when subjected to both ambient and accelerated stability testing. Less than 0.1 % degradation of ibuprofen is observed upon exposure over several months to all of the following environmental conditions:

- Ambient temperature and humidity,
- Ambient temperature, 100 % relative humidity,
- 37 °C and 60 °C, ambient relative humidity,
- 37 °C, 100 % relative humidity, and
- Ultra violet light, ambient temperature (Higgins *et al.*, 2001:292).

In the solution phase, ibuprofen has been shown to be relatively stable, even when exposed to harsh conditions such as 1.0 N NaOH, 1.0 N HCl or 50 % H_2O_2. Degradants that form in quantities less than 0.1 % include *iso*butylacetophenone (IBAP) and 2-(4-*iso*butyrylphenyl)-propionic acid (Higgins *et al.*, 2001:293).

2.7.4 Pharmacokinetics and metabolism

Ibuprofen is rapidly absorbed after oral administration and peak concentrations in plasma are observed after 15 to 30 minutes. The half-life in plasma is approximately 2 hours. Ibuprofen is extensively (99 %) bound to plasma

proteins, but the drug occupies only a fraction of the total drug-binding sites at usual concentrations. The excretion of ibuprofen is rapid and complete. More than 90 % of an ingested dose is excreted in the urine as metabolites or their conjugates. The major metabolites are a hydroxylated and a carboxylated compound (Jackson Roberts II and Morrow, 2001:711).

2.7.5 Pharmacology

It is well established that the S(+) enantiomer is almost entirely responsible for the anti-inflammatory effects of ibuprofen. The main mechanism of action is known to be the inhibition of prostanoid biosynthesis via blockade of cyclo-oxygenase (COX) (Higgins *et al.*, 2001:269). The COX enzyme has been shown to exist as two isoforms. COX-1 is a constitutive protein present in a wide range of cells and is important in the regulation of prostaglandins that are involved in the protection of the lining of the GI tract from noxious agents. It is thought that inhibition of COX-1 contributes to gastric ulceration, which is one of the most frequent side effects of NSAID therapy. COX-2 is the inducible form of the enzyme that is expressed in macrophages and other immuno-regulatory cells after trauma. Hence, the COX-2 isoform plays an important role in the inflammatory process (Higgins *et al.*, 2001:269).

2.7.6 Toxic effects

Although ibuprofen has been found to inhibit both COX isoforms *in vitro*, it still displays an acceptable safety profile. While ibuprofen has been shown to be relatively safe in doses ranging up to 2400 mg/day, typical effective oral doses range from 600 to 1800 mg/day (Higgins *et al.*, 2001:269). Ibuprofen has been used in patients with a history of gastrointestinal intolerance to other NSAIDs. Nevertheless, therapy usually must be discontinued in 10 to 15 % of patients because of intolerance to the drug. GI side effects are experienced by 5 to 15 % of patients taking ibuprofen. Epigastric pain, nausea, heartburn and sensations

of "fullness" in the GI tract are the usual difficulties (Jackson Roberts II and Morrow, 2001:712).

2.8 CONCLUSION

Chitosan is a versatile polymer with a wide range of applications. Chitosan has been shown to improve the dissolution rate of poorly soluble drugs and thus can be exploited for bioavailability enhancement of such drugs. The physical and chemical properties of chitosan, such as inter- and intra-molecular hydrogen bonding and the cationic charge in acidic medium, makes this polymer attractive for the development of conventional and novel pharmaceutical products. Reacting chitosan with controlled amounts of multivalent anion results in crosslinking between chitosan molecules. This crosslinking has been used extensively for the preparation of chitosan microspheres for different applications. The particle size of chitosan beads can easily be modified to give large surface to volume ratios, increasing the effective surface area of the drug incorporated and enhancing the dissolution rate of the drug from the delivery system. Chitosan beads further also prolongs the intestinal retention time by exhibiting mucoadhesive properties and floating on the intestinal fluid. Drugloading and the release of drugs from chitosan beads is dependant upon the molecular weight of chitosan, concentration of chitosan, drug content and density of crosslinking.

Ibuprofen is a non-steroidal anti-inflammatory drug (NSAID) with poor solubility and many gastrointestinal side effects (e.g. gastric ulceration). Incorporating ibuprofen in chitosan beads might reduce the epigastric side effects of the drug. Adding TMC, an absorption enhancer at neutral environments (pH 6.8), might also increase the drug bioavailibility within the small intestine. The following chapters will deal with the synthesis of a high and medium M_W TMC, the development of a chitosan microbead containing ibuprofen and TMC and the dissolution and *in vitro* evaluation thereof.

CHAPTER 3

SYNTHESIS AND CHARACTERISATION OF *N*-TRIMETHYL CHITOSAN CHLORIDE

Macromolecular drugs are poorly absorbed across mucosal membranes due to their potential hydrophilic nature and high molecular mass. As described in chapter 1, the co-administration of effective absorption enhancers may be a promising approach for the formulation of non-parenteral delivery systems for compounds with poor permeability through intestinal membranes. Chitosan is a non-toxic, biocompatible polymer that has found a number of applications in drug delivery including that of enhancing the absorption of hydrophilic drugs. Chitosan, when protonated (pH < 6.5), is able to increase the paracellular permeability of drugs across mucosal epithelia. Unfortunately chitosan is insoluble at neutral and alkaline pH values and therefore it is necessary to increase the solubility of these derivatives at these pH's in order to evaluate their effectiveness at neutral pH's such as those found in the intestinal tract. To increase the solubility of chitosan, partial quaternisation of the polymer was proposed and several methods have been investigated (Domard *et al.*, 1986:105).

N-trimethyl chitosan chloride (TMC) has been synthesised at different degrees of quaternisation by controlling the number and duration of reaction steps during the reductive methylation of chitosan as described by Sieval *et al.* (1998:157). During the reductive methylation of chitosan the primary amino groups on the C-2 position are changed to quaternary amino groups by substitution of the hydrogen atoms with methyl groups. The alkylation of the primary amine to the quaternary stage is simplified by using a base to bind the acid generated during the reaction and to avoid protonation of the unreacted primary amino groups (Domard *et al.*, 1986:105). This quaternised polymer forms complexes with anionic macromolecules and gels or solutions with cationic or neutral compounds

in aqueous environments and neutral pH values. TMC has been shown to considerably increase the permeation and/or absorption of hydrophilic compounds across intestinal epithelia in a neutral environment. The mechanism by which TMC enhances intestinal permeability is similar to that of protonated chitosan in that it reversibly interacts with components of the tight junctions, leading to widening of the paracellular routes (Kotzé *et al.*, 1999a:253).

3.1 SYNTHESIS OF TMC POLYMERS WITH DIFFERENT DEGREES OF QUATERNISATION

3.1.1 Materials

Chitoclear™ high molecular weight (97.6 % deacetylated chitosan, M_W = 263 700 g/mole) and Chitoclear™ medium molecular weight (93.2 % deacetylated chitosan, M_W = 77 670 g/mole) was a generous gift from Primex (Siglufjordur, Iceland) (annexures 1 and 2). All the reagents were obtained from Merck (South Africa) except for *N*-methyl-2-pyrrolidinone, which was purchased from Aldrich (South Africa), were of reagent grade and used as received during the synthesis. All water used was distilled and deionised. Apparatus used included a standard laboratory Liebig condenser, magnetic stirrer and waterbath.

3.1.2 Method

Two TMC polymers were synthesised in a two-step reaction according to the method of Sieval *et al.* (1998:157) from high and medium molecular weight Chitoclear™ chitosan respectively. Experimental conditions were kept similar for the synthesis procedures of both polymers.

First reaction step: A mixture of 2 g chitosan, 4.8 g sodium iodide, 11 ml of a 15 % (w/v) aqueous sodium hydroxide (NaOH) solution and 11.5 ml of methyl iodide in 80 ml of *N*-methyl-2-pyrrolidinone was stirred on a water bath at a temperature

of 60 °C for 45 minutes. A Liebig condenser was used to keep the methyl iodide in the reaction. The product was precipitated with ethanol and isolated by centrifugation.

Second reaction step: After washing the product (obtained from step one) with ethanol it was dissolved once again in 80 ml of *N*-methyl-2-pyrrolidinone. In this step 4.8 g of sodium iodide, 11 ml of a 15 % (w/v) aqueous sodium hydroxide solution and 7 ml of methyl iodide was added to the mixture. The mixture was stirred on a water bath at a temperature of 60 °C for 30 minutes.

Later adding step: Before the product was precipitated at the end of the reaction step an additional 2 ml of methyl iodide and 0.6 g of NaOH pellets was added to the reaction mixture. The mixture was stirred for another 45 minutes under the same conditions after which the product was precipitated with ethanol. To exchange the iodide with chloride the product was dissolved in 40 ml of a 5 % (w/v) aqueous sodium chloride (NaCl) solution and once again precipitated with ethanol. To remove any residual NaCl the polymer was dissolved in 40 ml of water and the precipitation step was repeated. The final product was washed twice with ethanol and diethyl ether and dried overnight under vacuum at 25 °C.

3.2 CHARACTERISATION OF TMC POLYMERS

The synthesised TMC polymers were characterised by using nuclear magnetic resonance (NMR) spectrometry, determination of the molecular weight by size exclusion chromatography connected to a multi-angle laser light scattering apparatus (SEC/MALLS with a laser photometer and refracting index detector), infrared spectrometry (IR) analysis and measurement of the intrinsic mucoadhesivity. These analytical techniques were used to determine differences in the molecular weight and the intrinsic mucoadhesivity of the various polymer solutions and to calculate the exact degree of quaternisation of each polymer.

3.2.1 Methods

3.2.1.1 Nuclear magnetic resonance (NMR) spectroscopy

Proton NMR (^{1}H-NMR) is a form of absorption spectrometry (related to infrared and ultraviolet spectrometry) and is based on the theory that under appropriate conditions in a magnetic field, a sample can absorb electromagnetic radiation in the radio frequency region at frequencies governed by the characteristics of the sample. Absorption is a function of certain nuclei in the molecule. A plot of the frequencies of the absorption peaks versus peak intensities constitutes a NMR spectrum (Silverstein *et al.*, 1991:165).

The ^{1}H-NMR spectrum was recorded in D_2O with a Bruker 300 MHz spectrometer (Bruker, Karlsruhe, Germany). The percentage degree of quaternisation of each polymer was calculated from the results of the ^{1}H-NMR spectra using the following equation (Sieval *et al.*, 1998:158):

$$DQ\ (\%) = \left[\left(\frac{\int TM}{\int H}\right) x\ \frac{1}{9}\right] x\ 100 \qquad (1)$$

where:

DQ (%) = degree of quaternisation expressed as a percentage,

$\int$TM = integral of the trimethyl amino group peak, and

$\int$H = integral of the ^{1}H peaks.

3.2.1.2 Determination of the molecular weight

Multi-angle laser light scattering instruments are able to determine absolute molar masses of polymers and biopolymers from below a thousand Dalton (g/mole) to hundreds of millions of Daltons. The light scattering instrument measures the absolute molecular weight based on the following method:

A vertically polarised laser beam passes through the sample, either in a flow-through cell or in a static (batch) container. The sample of polymer macromolecules scatters light into all angles. Each detector, which is placed at a different angular position around the sample, provides a response directly proportional to the intensity of the scattered light it receives. The analogue light scattering signals are digitised and transmitted to a computer for processing. The computer software performs the analysis necessary to extract the absolute molecular weight from the data (Wyatt Technology, 1999:4).

The absolute molecular weight of the synthesised TMC polymers were measured with a size exclusion chromatograph (SEC) (Hewlett Packard 1100, USA) connected to a multi-angle laser light scattering detector (MALLS) consisting of a photometer (Dawn DSP, Wyatt Technology Corporation, Santa Barbara, CA, USA) coupled to an ERC 7515 A refractive index detector (ERC Inc., Tokyo, Japan) as previously described by Snyman *et al.* (2002:145).

The polymers were dried under vacuum for a period of 24 hours at a temperature of 40 °C. Aqueous solutions (5 mg/ml) suited for chromatography were prepared. A volume of 0.8 ml of these solutions were filtered using 0.2 µm membrane filters (Sartorius AG, Germany) and collected in chromatographic sample vials. The mobile phase consisted of 0.2 M ammonium acetate (Saarchem, South Africa) and the pH was adjusted to 4.5 with acetic acid (Saarchem, South Africa). The experimental apparatus consisted of a HP 1100 vacuum degasser, isocratic pump and auto sampler connected to a TSK-guard PWH inline column (Toso Haas, Japan). The size exclusion columns included a TSK G6000 PW column (Toso Haas, Japan, inside diameter = 7.5 mm, length = 30 cm, particle size > 17 µm, pore size > 1000 Å) connected in series with a TSK G5000 PW column (Toso Haas, Japan, inside diameter = 7.5 mm, length = 30 cm, particle size = 17 µm, pore size = 1000 Å). Samples of 100 µl were injected at a flow rate of 0.8 ml/min and were analysed with the laser photometer (He/Ne laser, λ = 633 nm) and a refracting index detector (ERC 7515 A, Japan). The

data from the detector was integrated with a computer using Astra® for Windows software (Wyatt Technology Corporation, Santa Barbara, CA, USA).

3.2.1.3 Infrared (IR) spectrometry

Infrared radiation in the range from about 10 000 to 100 cm^{-1} is absorbed and converted by an organic molecule into energy of molecular vibration. This absorption is quantised and these vibrational spectra appear as bands. The frequency or wavelength of absorption depends on the relative masses of the atoms, the force constants of the bonds and geometry of the atoms. Molecular vibrations appear as stretching and bending vibrations. Only those vibrations that result in a rhythmical change in the dipole moment of the molecule are observed in IR (Silverstein *et al.*, 1991:91).

IR spectra were recorded on a Nicolet Magna IR 550 series II spectrometer (Nicolet Analytical Instruments, Madison, WI) over a range of 4000 to 400 cm^{-1} using the potassium bromide disc technique. Samples of the TMC polymers weighing approximately 2 mg were mixed with 200 mg of KBr (Merck, South Africa) with a mortar and pestle. This mixture was filled in an infrared press and compressed for approximately 1 minute to form a disc.

3.2.1.4 Determination of the intrinsic mucoadhesivity

The mucoadhesive properties of the TMC polymers and their parent chitosans were determined according to a method of Snyman *et al.* (2003:59). The experimental set-up for the tensile separation apparatus is shown in figure 3.1 and was based on an adaption of the Wilhelmy plate method in which the mucoadhesion strength of the bond between mucus and the polymers was measured. Pectin from apple (USP) (Sigma, UK) and a clean plate were used as reference standards.

Solutions of the polymers were made by dissolving 0.1 g of each polymer in 10 ml aqueous glacial acetic acid (2 % v/v) (1 % w/v polymer solution). The aluminium plates were prepared by adding 0.5 ml of the solution on each plate to produce 5 mg of polymer film after drying. The prepared aluminium plates were suspended from a microbalance (Hugo Sachs Elektronik, Force Transducer F30 Type 372, Germany) and the plates were lowered until contact was achieved with a 30 % (w/v) solution of mucus (partially purified porcine gastric mucin type III, Sigma, UK) in distilled water at 25 °C. The plate was lowered until the tension between the plate and the microbalance declined, ensuring a 2 g downward pressure of the plate on the mucus. The plate was left in this position for different time intervals (30, 60, 90 and 120 seconds) for hydration of the polymer to occur, after which it was once again lifted at a rate of 0.25 mm/s. The separation was registered by software (Chart for Windows v3.4, Powerlab System, Oxfordshire, UK) and the maximum detachment force (MDF) was noted. All experiments were performed in six-fold at room temperature (25 °C).

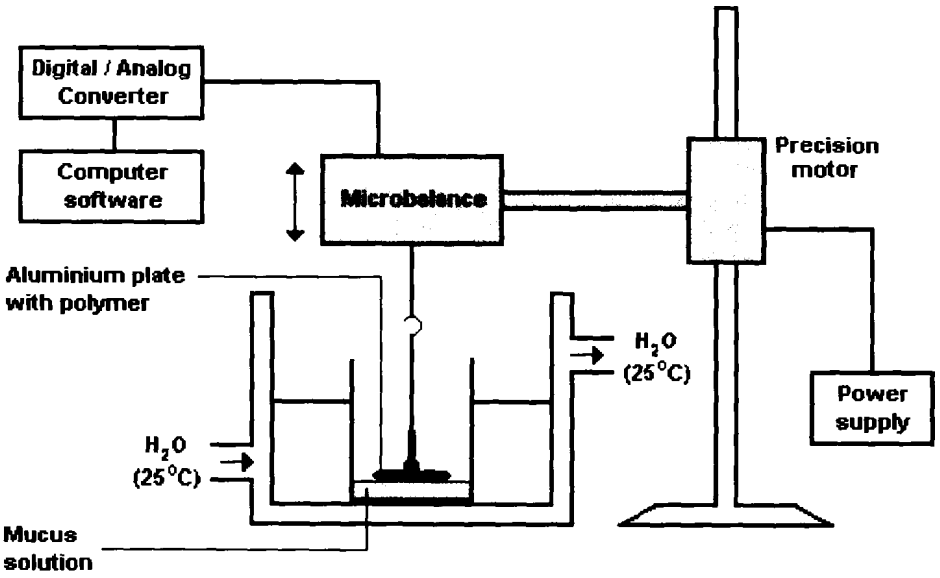


FIGURE 3.1 Apparatus used for the tensile separation testing procedure.

To determine the intrinsic mucoadhesivity (IM) of the polymers the maximum detachment force was calculated at a time of 120 seconds. The IM was used to rank the polymers according to their mucoadhesive strength. Pectin was chosen as the 100 % reference to which the synthesised TMC polymers and their parent chitosans were compared. The baseline (clean plate reference standard) was subtracted from the IM value of each of the polymers and the percentage mucoadhesivity of the polymer to pectin was calculated.

3.2.2 Results

3.2.2.1 Nuclear magnetic resonance (NMR) spectroscopy

The ^{1}H-NMR spectra for the medium molecular weight TMC (TMC-M) synthesised from medium molecular weight Chitoclear™ (93.2 % deacetylated chitosan, M_w = 77 670 g/mole) and the high molecular weight TMC (TMC-H) synthesised from high molecular weight Chitoclear™ (97.6 % deacetylated chitosan, M_W = 263 700 g/mole) is depicted in figures 3.2 and 3.3. Sieval *et al.* (1998:158) has assigned the peak at 3.1 ppm to dimethyl amino groups and the peak at 3.3 ppm to the trimethyl amino groups for the ^{1}H-NMR spectra of TMC. The peaks between 4.7 and 5.7 ppm were assigned to the ^{1}H protons. The integrals of the peaks were substituted into equation 1 to calculate the degree of quaternisation for the synthesised polymers. The degree of quaternisation calculated for the TMC-H and TMC-M polymers were 48.5 % and 74.7 % respectively.

Since the experimental conditions were the same during the synthesis of both TMC-H and TMC-M, the difference in quaternisation between the two polymers might be explained by the difference in the molecular weights of their respective parent chitosans. It was hypothesised that the long chains of the high molecular weight chitosan favours entanglement/coiling of the polymer chains. This decreases the availability of the primary amino groups on the C-2 position of chitosan for reductive methylation. The medium molecular weight chitosan has

shorter chain lengths with less entanglement/coiling and access to the C-2 position are therefore easier. This might lead to the TMC-H polymer having a lower degree of quaternisation than the TMC-M derivative. Little difference also exists in the degree of deacetylation of the parent chitosans.

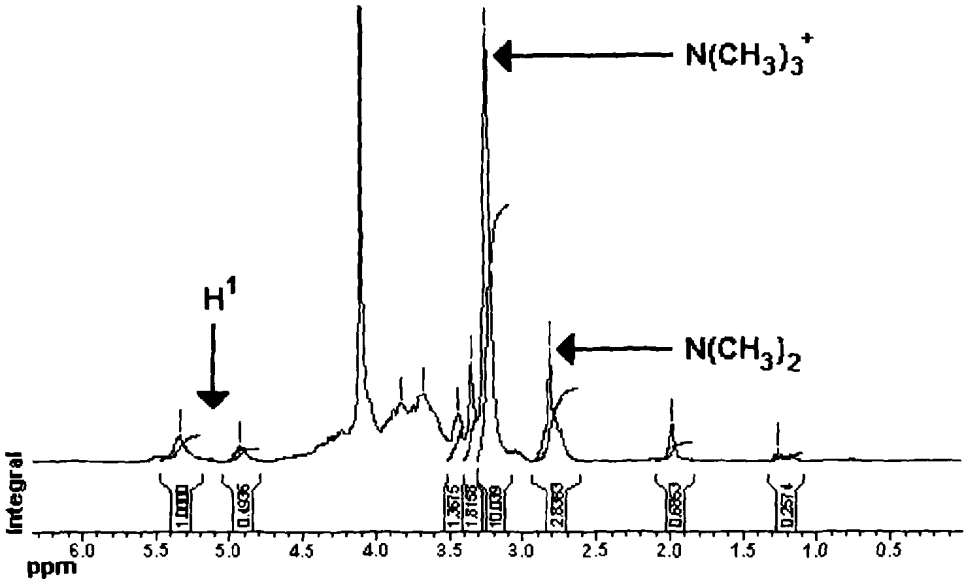


FIGURE 3.2 ^{1}H-NMR spectrum for the medium molecular weight TMC (TMC-M) synthesised from medium molecular weight Chitoclear™.

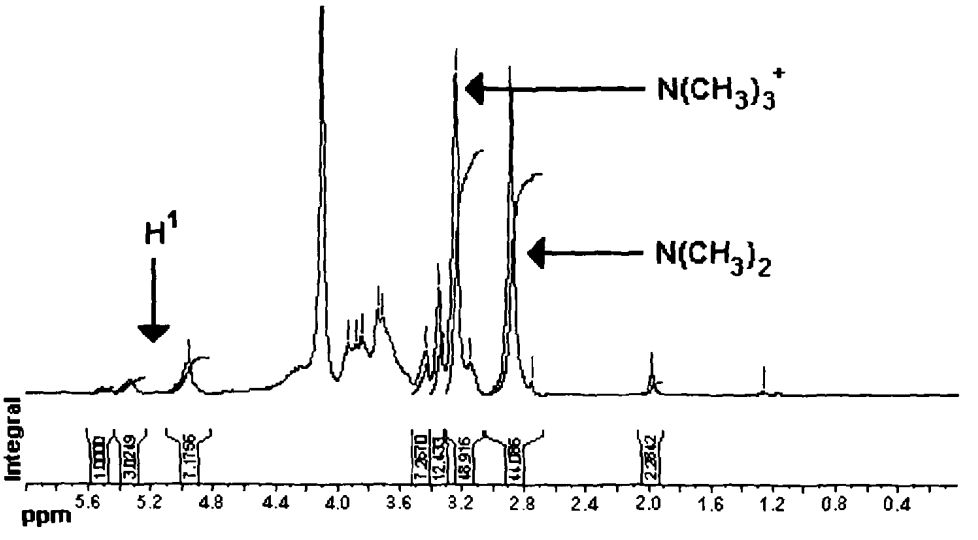


FIGURE 3.3 ^{1}H-NMR spectrum for the high molecular weight TMC (TMC-H) synthesised from high molecular weight Chitoclear™.

3.2.2.2 Determination of the molecular weight

To clarify the results obtained, the terms *molecular mass* and *molecular weight* need to be defined. *Molecular mass* refers to the mass of a quantity of one mole of subunits (monomers) of the polymer and addition of groups or molecules to these subunits should increase the molecular mass. *Molecular weight* in this context is the average weight of the polymer chain (monomers linked to each other to form a polymer) and may be affected by degradation of the TMC polymer during the synthesis process.

The mean molecular weights of the synthesised TMC-M and TMC-H polymers were respectively 64 100 g/mole and 233 700 g/mole as determined by SEC/MALLS. Although the molecular weight of the polymer chain should increase during the methylation procedure, a net reduction in molecular weights was observed. The reduction is attributed to the reaction conditions during the synthesis (strong alkaline environment and elevated temperature) which are probably responsible for degradation of the chain length of the polymer molecules.

3.2.2.3 Infrared (IR) spectrometry

Infrared spectra of the synthesised TMC polymers are depicted in figure 3.4. According to Silverstein *et al.* (1991:124) medium to weak absorption bands for C-N linkage in aliphatic amines appears in the region of 1250 to 1020 cm^{-1}. The vibrations responsible for these bands involve C-N stretching coupled with the stretching of adjacent bonds in the molecule. Strong medium absorption bands can be seen at 1062 cm^{-1} on the infrared spectra in figure 3.4 for both the TMC polymers. It is expected that the intensity of this absorption band should increase for TMC polymers with higher degrees of quaternisation indicating an increase in the concentration of C-N bonds in the compound and therefore also increase in the number of methyl groups added to the amino groups of the TMC molecule.

However, the result obtained from the IR analysis seems to be conflicting, as the intensity of the absorption band for both TMC-M and TMC-H seems to be concurrent. This might be explained by the longer polymer chain length of TMC-H which probably has increased numbers of amino groups present relative to that of the TMC-M and therefore the actual amount of methyl groups added to these amino groups are increased as well. Therefore, although TMC-M (higher degree of quaternisation) should theoretically exhibit a more intense absorption band at 1062 cm^{-1}, it is possible for the absorption band of TMC-H (longer polymer chain length) to coincide with that of TMC-M.

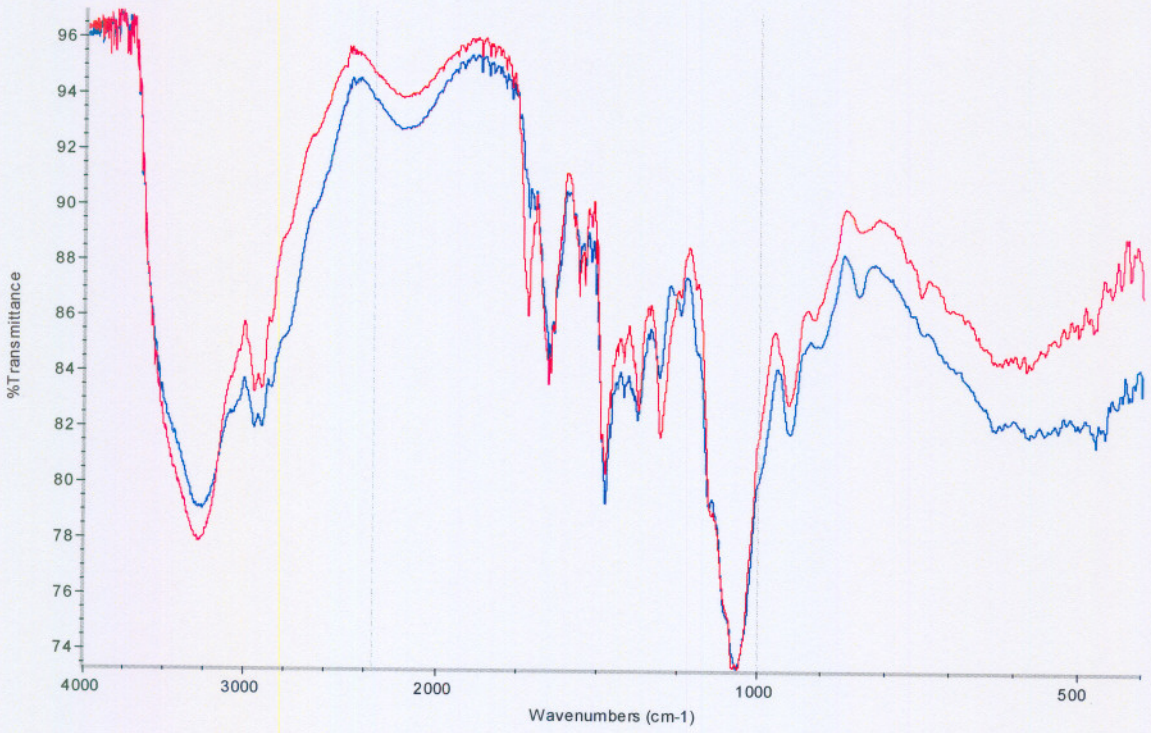


FIGURE 3.4 The intensity of infrared absorption as a function of the wavenumber of TMC-M (■) and TMC-H (■).

3.2.2.4 Determination of the intrinsic mucoadhesivity

Figure 3.5 shows an example of a typical result from the tensile detachment testing of one of the polymers. The increase in the force applied as a function of time was determined in the experiment and the maximum detachment force

(MDF) was taken as an indication of the mucoadhesion strength of the polymer at the given time. The tensile force increases as the plate was lifted until the bond between the plate and the mucus was broken (maximum detachment force) after which the tensile force decrease. The amount of mucus still adhering to the plate can be seen by the difference in the baseline observed before and after the experiment and is an indication that breakage of the interface occurred nearer to the side of the mucus.

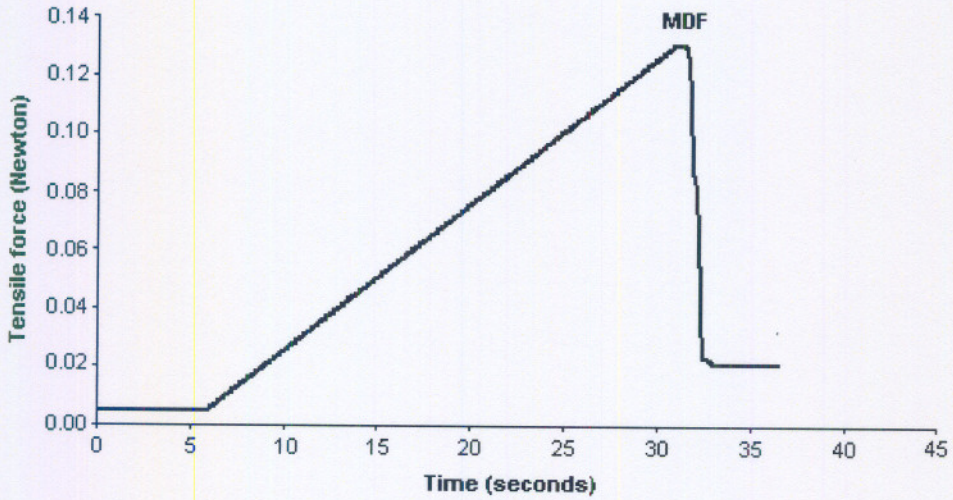


FIGURE 3.5 A typical result of tensile detachment testing with the tensile force (N) as a function of time (s).

The mucoadhesion profiles over a period of 120 seconds for the two synthesised TMC polymers (TMC-M, TMC-H), their parent chitosans (Chit-M, Chit-H) and pectin are shown in figure 3.6 with the clean plate reference as control. The chitosan (Fijian-000201) (annexure 3) used for the synthesis of the microbead delivery system in chapter 4 was also tested for mucoadhesive strength and included with the mucoadhesivity results. The mucoadhesion profiles for TMC-M and TMC-H corresponds well with the profiles of their respective parent chitosans, which indicates that the synthesis process had little effect on the

mucoadhesive strength. The difference between the medium molecular weight (TMC-M, Chit-M) and high molecular weight (TMC-H, Chit-H) polymers are also clear. The weaker mucoadhesivity displayed by the lower molecular weight polymers might be explained by slower penetration in the mucus layer because of sterical hindrance or intermolecular associations. This might be related to the higher degree of quaternisation and poor entanglement with mucin due to shorter chain lengths.

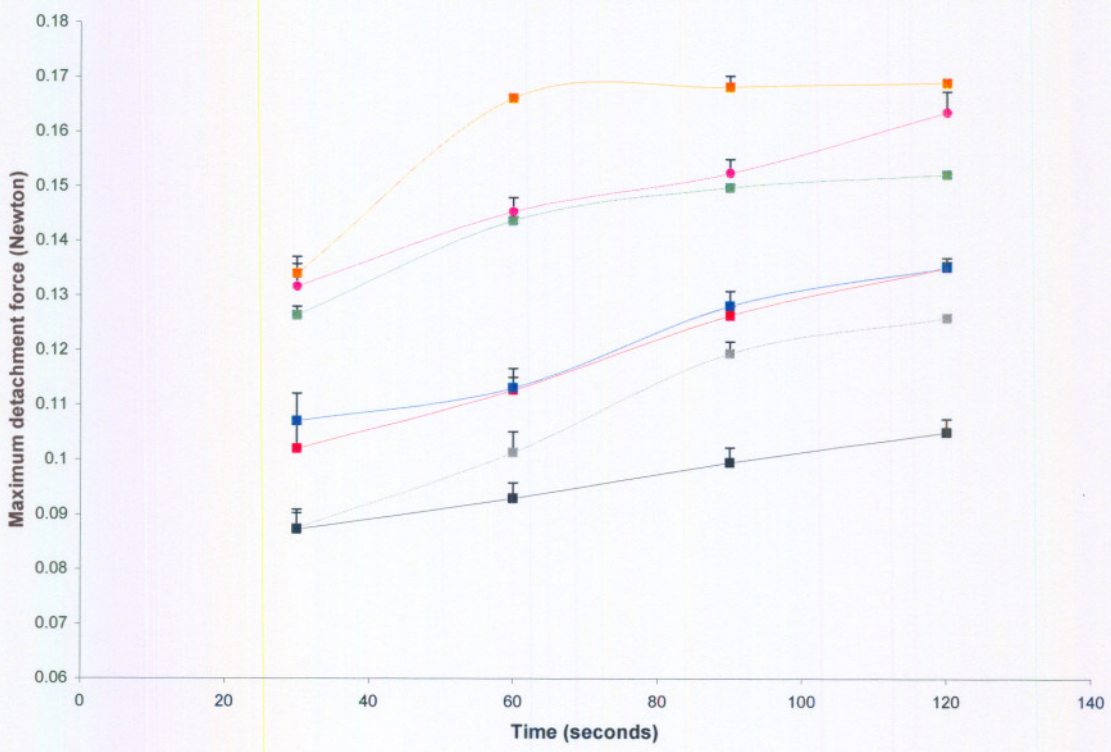


FIGURE 3.6 Mucoadhesion as a function of time for pectin (■), TMC-M (■), Chit-M (■), TMC-H (■), Chit-H (■) and Fijian000201 chitosan (■) with the clean plate (■) as control. Data is expressed as the mean ± SD of 6 experiments.

Figure 3.7 depicts the intrinsic mucoadhesivity (IM) for TMC-M, TMC-H, Chit-M, Chit-H and Fijian000201 chitosan as a percentage relative to pectin. Pectin exhibits relatively poor mucoadhesive properties, but is advantageous in that small deviations from the accepted values (obtained with different experiments) are obtained and it is therefore a suitable reference standard (Snyman *et al.*,

2003:65). A difference of 81.73 % between the IM of TMC-M (144.23 %) and TMC-H (225.96 %) was calculated. It is known that with an increase in the degree of quaternisation of TMC the mucoadhesive properties of the polymer are decreased (Snyman *et al.*, 2003:59). The difference of 125.96 % in the IM between TMC-H (225.96 %) and pectin (100.00 %) indicates that TMC-H exhibits relatively strong mucoadhesive properties, while the Fijian000201 chitosan (307.69 %) showed the strongest mucoadhesivity with a 207.69 % increase in IM over that of pectin (100.00 %).

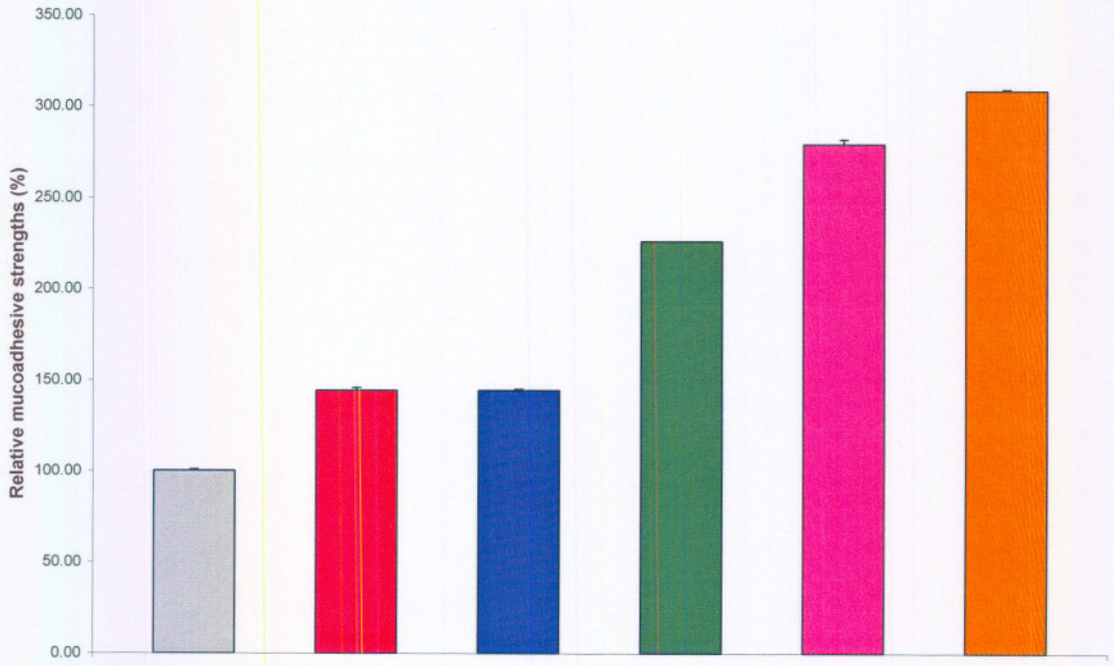


FIGURE 3.7 Intrinsic mucoadhesivity of pectin (■), TMC-M (■), Chit-M (■), TMC-H (■), Chit-H (■) and Fijian000201 chitosan (■). Data is expressed as the mean ± SD of 6 experiments.

3.3 CONCLUSION

Two TMC polymers with different degrees of quaternisation have been synthesised by reductive methylation with methyl iodide in the presence of sodium hydroxide by varying the molecular weight of the initial parent chitosan.

A medium molecular weight (TMC-M, M_W = 64 100 g/mole) and high molecular weight (TMC-H, M_W = 233 700 g/mole) TMC, with degrees of quaternisation of 74.7 % and 48.5 % respectively, were obtained. It was hypothesised that the long chains of the high molecular weight chitosan favours entanglement of the polymer chains, which decreases the availability of the C-2 position of chitosan for reductive methylation. Therefore, a lower degree of quaternisation was obtained for the TMC-H polymer. During synthesis the polymer chain undergoes degradation due to the strong alkaline environment and high temperatures. It was also shown that the molecular weight of the TMC polymers decreased due to this degradation.

It was expected that the extent of degradation is not enough to theoretically influence the mucoadhesive properties of TMC as the mucoadhesivity of both TMC polymers correlated well with that of their parent chitosans. TMC-H exhibits greater mucoadhesive strength than TMC-M due to an increase in the degree of quaternisation of TMC-M. The weaker mucoadhesivity displayed by the lower molecular weight TMC-M might be explained by slower penetration in the mucus layer because of sterical hindrance or intermolecular associations.

IR spectra confirmed an increase in the degree of quaternisation of TMC-M which corresponded with the higher polymer chain length of lower quaternised TMC-H. In the following chapter the synthesis and characterisation of a possible chitosan microbead delivery system will be described. Special emphasis will be placed on the loading and release characteristics of the synthesised TMC polymers as possible absorption enhancers and ibuprofen as model drug compound.

CHAPTER 4

PREPARATION AND CHARACTERISATION OF CHITOSAN MICROBEADS CONTAINING IBUPROFEN AND TMC

In chapter 1 the safety and absorption enhancing capabilities of chitosan and its *N*-trimethyl derivative (TMC) were described. Although TMC was proved to be a potent absorption enhancer of hydrophilic drugs, both *in vitro* and *in vivo*, it has always been administered as a solution (Kotzé *et al.*, 1999a:360; Thanou *et al.*, 2000b:951; Thanou *et al.*, 2001b:824). The basic principles of beads as a solid oral dosage form, the different techniques to prepare beads and the physico-chemical properties of ibuprofen were discussed in chapter 2. It was concluded that chitosan microbeads, containing TMC, show ample potential for the formulation of solid oral drug delivery systems with absorption enhancing properties of hydrophilic drug compounds in neutral environments.

Because of their water-absorbing capacity, microbeads are the subject of investigation for researchers interested in fundamental aspects of swollen polymeric networks. Microbeads are polymeric networks, which absorb and retain large amounts of water. In the network, hydrophilic domains are present which are hydrated in an aqueous environment, thereby creating a hydrogel structure. As the term 'network' implies, crosslinks have to be present to avoid dissolution of the hydrophilic polymer chains/segments into the aqueous phase. A great variety of methods (e.g. ionotropic gelation) to establish crosslinking has indeed been used to prepare hydrogel structures. Chitosan has one primary amino and two free hydroxyl groups for each C_6 building unit (figure 1.5, § 1.3.1.1). Due to the easy availability of free amino groups in chitosan, it carries a positive charge at lower pH values and thus in turn reacts with many negatively charged surfaces/polymers and also undergoes ionotropic gelation with

counterions such as pentasodium tripolyphosphate (TPP) (Bodmeier *et al.*, 1989:1475).

In chapter 3 the synthesis and characterisation of a medium (TMC-M) and high (TMC-H) molecular weight *N*-trimethyl chitosan chloride derivative was discussed. In chapter 4 the formulation and evaluation of different chitosan microbeads containing TMC-M (degree of deacetylation = 74.7 %) or TMC-H (degree of deacetylation = 48.5 %), ibuprofen and different quantities of disintegrating agents will be described. Commonly used modified cellulose gum (Ac-di-sol® (ADS)) and sodium starch glycolate (Explotab® (EXP)) were added as disintegrants to the different microbead formulations to promote release of both the drug and TMC from the beads. It was also hypothesised that an increased acidity of the micro-environment in the beads might improve the solubility of both chitosan and TMC. A resultant increase in water-absorbing capacity and increased TMC and drug release from the microbeads might therefore follow. Thus, ascorbic acid (AA) was included to investigate the effect of acidity on the different microbead formulations. Dissolution studies in phosphate buffered solution (PBS; pH 6.8) were performed on all microbead variants to determine the following:

- if chitosan beads are indeed a suitable carrier for TMC and ibuprofen,
- which TMC derivative (TMC-M or TMC-H) is the best candidate for loading into and release from chitosan microbeads, and
- which concentration of additive (Ac-di-sol®, Explotab® or ascorbic acid) shows the most favourable release of both TMC and ibuprofen.

Firstly, the effect of different concentrations TMC-M and TMC-H on chitosan microbeads was studied with results obtained from scanning electron microscopy (SEM), TMC loading capacity and microbead swelling behaviour. After selection of the most suitable TMC concentration, the effect of varying concentration (0.1, 0.2 and 0.5 %) additives on TMC and ibuprofen release was studied. Results

were obtained from SEM imaging, TMC and ibuprofen loading capacity, swelling behaviour and dissolution release profiles.

4.1 PREPARATION OF CHITOSAN MICROBEADS

4.1.1 Materials

The Fijian-000201 chitosan (83 % deacetylated) (annexure 3) were purchased from Edelweis Pharmaceuticals (Johannesburg, South Africa). The TMC-M and TMC-H used were synthesised (chapter 3) and ibuprofen (annexure 4) was purchased from Sandoz (Isando, South Africa). All other reagents were obtained from Merck Co. (Midrand, South Africa) except for pentasodium tripolyphosphate, which was purchased from Sigma (Johannesburg, South Africa) and were used as received. All water used was distilled and deionised. The polymer solution was transferred to a 10 % (w/v) aqueous TPP solution by a Watson Marlow peristaltic pump (Watson Marlow Ltd., Falmouth, England).

4.1.2 Method

Microencapsulation generally involves two steps: (1) drop formation (discrete droplet formation or emulsification), followed by (2) droplet solidification (gelation, membrane formation, or other means). The most widespread laboratory method is by extrusion of polymer droplets containing the drug through a needle, then into a gel forming solution, solidifying the falling droplets in microbeads or capsules. The main drawback of the droplet extrusion method is the large size (greater than 1 mm) of the resultant beads and the low rate of the bead formation (Poncelet *et al.*, 1999:2018). Microbeads with a diameter of 800 to 1200 µm were prepared by ionotropic gelation as adapted from Bodmeier *et al.* (1989:1478). The decrease in droplet diameter was obtained by applying airflow across the extruding polymer droplets as shown in figure 4.1. Chitosan (Fijian-000201; 3 % w/v), ibuprofen (2 % w/v), TMC (1, 2 or 3 % w/v) and Ac-di-sol®,

Explotab® or ascorbic acid (0.1, 0.2 or 0.5 % w/v) was dissolved in 2 % (v/v) aqueous glacial acetic acid and stirred for 12 hours to obtain transparent, homogeneous solutions. The solutions were then left standing for 30 minutes to allow the air bubbles to disperse.

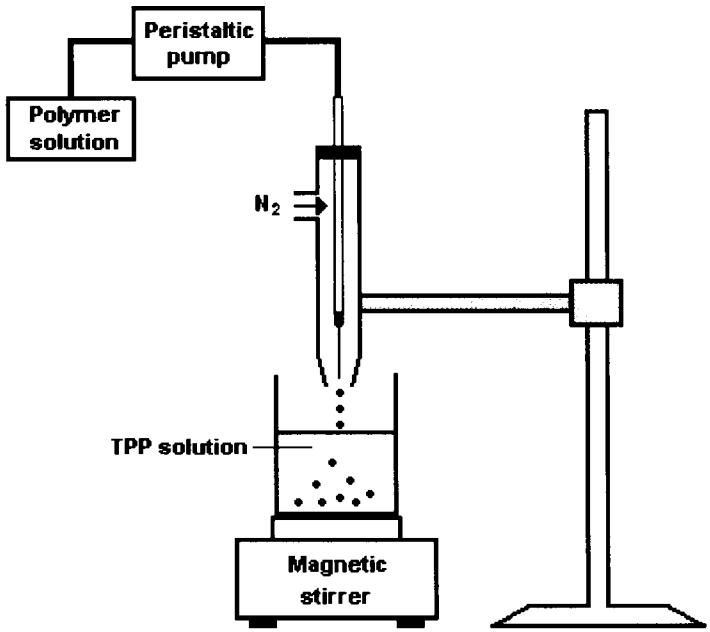


FIGURE 4.1 Schematic illustration of the apparatus used for the preparation of chitosan microbeads.

A 10 % (w/v) aqueous TPP solution was prepared and the pH adjusted to pH 4.2 with 1.0 M aqueous hydrochloric acid solution (Win *et al.*, 2003:306). The TPP solution was stirred until the aqueous solution became clear. 20 ml of the various polymer solutions was then pumped with a peristaltic pump through a gauge 18 needle into 100 ml of the gently agitated TPP solution, while nitrogen gas was blown over the extruding polymer droplets. The airflow was regulated with a Spectrol Special Gases regulator (Afrox, South Africa) at a constant pressure of 20 kPa in order to obtain a mean microbead size diameter of approximately 1000 µm. The microbeads were formed on contact with the TPP solution. A stirring time of 30 minutes was allowed for crosslinking before the microbeads were removed from the TPP solution by filtration. The microbeads

were then washed twice with 50 ml deionised water to remove any excess TPP and ibuprofen from the surface of the beads. Beads were prepared at room temperature before being frozen at -70 °C and lyophilised for 12 hours in a Secfroid freeze-dryer (Lausanne, Switzerland). The weight of dry microbeads for each synthesis was recorded for use to determine the drugloading. A schematic diagram for the preparation of ibuprofen- and TMC-loaded chitosan microbeads is shown in figure 4.2. Synthesised microbeads were subsequently characterised by SEM imaging, TMC and ibuprofen loading, swelling behaviour analysis and dissolution studies.

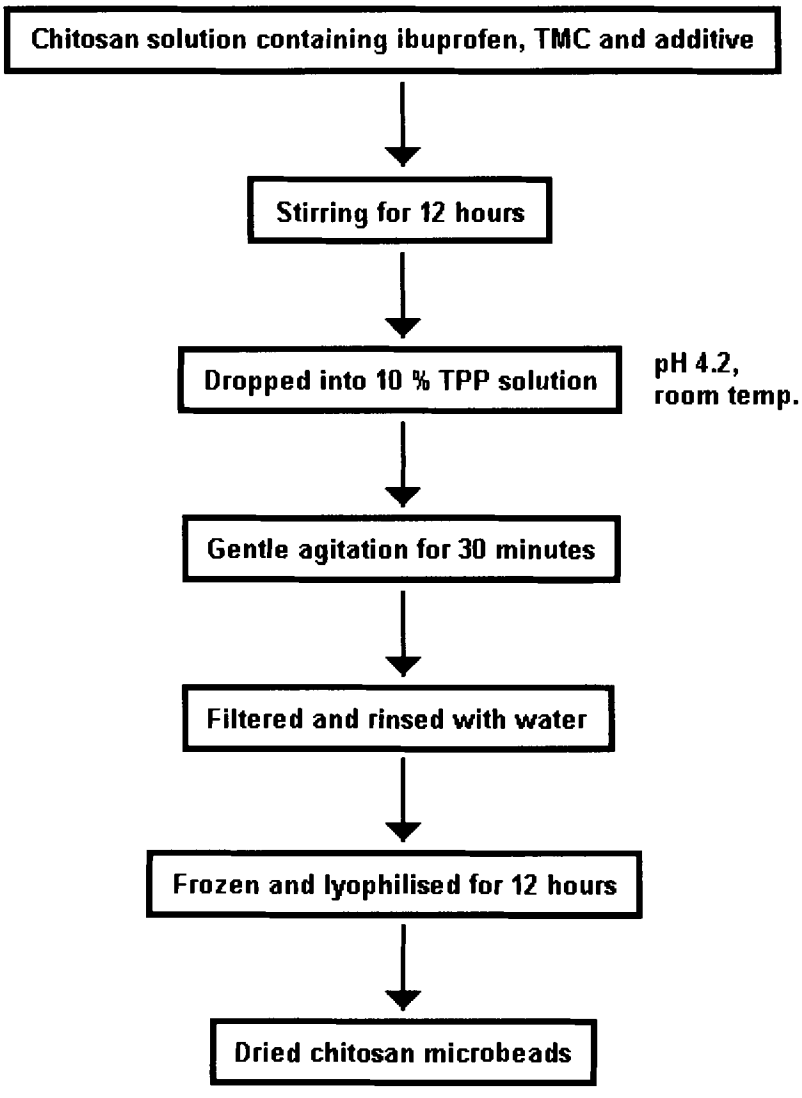


FIGURE 4.2 A schematic diagram for the preparation of ibuprofen- and TMC-loaded chitosan microbeads.

4.2 CHARACTERISATION OF CHITOSAN MICROBEADS

4.2.1 Methods

Eighteen variations of chitosan microbeads (table 4.1) were synthesised and compared with (1) chitosan microbeads containing only ibuprofen (CHIT), (2) chitosan microbeads containing ibuprofen and TMC-M (CHIT-M) and (3) chitosan microbeads containing ibuprofen and TMC-H (CHIT-H) as controls. The controls were identical to the different variations of microbeads synthesised, differing only in the additives added. The following experimental studies for purposes of characterisation were performed on all twenty-one formulations:

4.2.1.1 Morphology

Morphology studies on all microbead variants were performed with scanning electron microscope (SEM) imaging. For each variant a surface and cross-section micrograph were taken to determine the size, shape and porosity of the microbead. Samples were mounted on an aluminium sample mount and coated under vacuum with carbon before being sputter-coated with gold-paladium alloy to minimise surface charging. SEM analysis was performed with a FEI-Quanta 200 scanning electron microscope (FEI, USA).

4.2.1.2 Determination of TMC and ibuprofen loading

The TMC and ibuprofen loading of the microbeads were determined by an indirect method during the preparation of the beads. After removal of the microbeads, the water used to wash the excess TPP and ibuprofen from the bead surface was added to the remaining TPP solution. A sample was taken and the concentration TMC and ibuprofen were respectively determined by ultraviolet (UV) spectrophotometric analysis.

TABLE 4.1 Classification of the different chitosan microbead formulations containing either TMC-M or TMC-H and varying concentrations (0.1, 0.2 or 0.5 % w/v) of additives.

DIFFERENT CHITOSAN MICROBEAD FORMULATIONS		
TMC-M	Ac-di-sol® (ADS)	M-ADS-0.1
		M-ADS-0.2
		M-ADS-0.5
	Explotab® (EXP)	M-EXP-0.1
		M-EXP-0.2
		M-EXP-0.5
	Ascorbic acid (AA)	M-AA-0.1
		M-AA-0.2
		M-AA-0.5
TMC-H	Ac-di-sol® (ADS)	H-ADS-0.1
		H-ADS-0.2
		H-ADS-0.5
	Explotab® (EXP)	H-EXP-0.1
		H-EXP-0.2
		H-EXP-0.5
	Ascorbic acid (AA)	H-AA-0.1
		H-AA-0.2
		H-AA-0.5

To clarify the loading results obtained, the terms *percentage drug loading capacity* (DLC) and *percentage drug content* (DC) need to de defined. DLC refers to the percentage of drug recovered from the original amount introduced to each synthesis. DC in this context is the percentage of drug present per dry weight of beads and is determined from the DLC and dry weight of each synthesis.

Since a known volume of polymer solution were added to the TPP solution, the amount of TMC or drug lost in the TPP solution and during the washing process can be calculated. By subtracting the amount of TMC or drug lost to the TPP solution during the preparation of the microbeads from the initial amount in the polymer solution, the percentage DLC can be calculated by the following equation:

$$\%\ \text{Drug loading capacity (DLC)} = \left[\frac{(M_o - M_s)}{M_o}\right] \text{x } 100 \qquad (2)$$

where:

M_o = the original amount (mg) of TMC or drug in the prepared solution,

M_s = the amount (mg) of TMC or drug determined in the TPP solution and water used to wash the beads.

Further, to allow for correct dosing of either TMC or ibuprofen, one needs to determine the percentage TMC or drug present per dry weight of beads (DC) for each individual formulation. The percentage DC can thus be calculated with the following equation:

$$\%\,\text{Drug content (DC)} = \left[\frac{(\%\,\text{DLC x } M_o)/100}{M_d}\right] \text{x} 100 \qquad (3)$$

where:

% DLC = the percentage drug loading capacity calculated from equation 2,

M_o = the original amount (mg) of TMC or drug in the prepared solution,

M_d = the dry weight of synthesised beads for each individual synthesis.

The % DLC and the resultant % DC were calculated for the synthesis of each individual chitosan microbead formulation (table 4.1). Only one synthesis for each formulation of microbead was performed and therefore no mixing of different batches of the same variant occurred.

4.2.1.2.1 Colorimetric assay of TMC

A colorimetric method for determining chitosan in an aqueous solution was described by Muzzarelli (1998:255). This method was used as the basis for the TMC-M and TMC-H colorimetric assays. A solution of Cibachron brilliant red 3B-A (Aldrich, USA) was prepared in deionised water at a concentration of 1.5 mg/ml. Aliquots (5ml) of the stock solution were made up to 100 ml with phosphate buffer solution (PBS) at pH 6.8 as prepared according to the USP (2004:2724). The final concentration of the dye solution was 75 µg/ml.

To prepare the standard curve, a stock solution of TMC was prepared with a concentration of 400 µg/ml. Standard solutions with concentrations ranging from 80 to 240 µg/ml were prepared by pipetting different volumes of the stock solution directly into disposable cuvettes, after which the volume in each cuvette was filled up to 1000 µl with PBS (pH 6.8). Aliquots of the dye solution (2 ml) were added to each cuvette and the absorbance was recorded within 5 minutes, after adding the dye, at a wavelength of 572 nm with a Varian Cary 50 Conc UV-Visible spectrophotometer coupled to CaryWinUV Ver 3.0 software (Varian, USA). Samples were stored for a maximum period of six hours at ambient temperature prior to analysis and standard curves were obtained each day of analysis. Samples, obtained from the synthesis of microbeads and the

dissolution studies, were analysed in a similar way. All the standard curves exhibited a Lambert-Beer's law relationship in the concentration range employed, with correlation coefficients (R^2) of ≥ 0.994.

4.2.1.2.2 UV spectrophotometric analysis of ibuprofen

A validate spectrophotometric analysis for determining ibuprofen in an aqueous solution was used as previously described by du Preez (2002:38). Standard solutions with concentrations ranging from 10 to 50 µg/ml were prepared from a stock solution containing 125 mg ibuprofen dissolved in approximately 70 ml absolute ethanol and PBS (pH 6.8) to 250 ml. The stock solution was placed in an ultrasonic bath for 5 minutes to ensure complete solution of the drug.

The UV-absorbancies of the standard solutions were determined in triplicate at 225 nm against PBS (pH 6.8) as blank, using a Varian Cary 50 Conc UV-Visible spectrophotometer coupled to CaryWinUV Ver 3.0 software (Varian, USA). Samples were stored for a maximum period of six hours at ambient temperature prior to analysis and standard curves were obtained each day of analysis. Samples obtained from the synthesis of microbeads and the dissolution studies were analysed in a similar way. All the standard curves exhibited a Lambert-Beer's law relationship in the concentration range employed, with correlation coefficients (R^2) of ≥ 0.999.

4.2.1.3 Swelling behaviour analysis

Swelling behaviour studies on all chitosan microbead variations were performed according to a method published by Win *et al.* (2003:306). Dried chitosan microbeads were carefully weighed and immersed in 3 ml PBS solution with a pH of 6.8. The temperature was kept constant in a waterbath at 37 °C. At predetermined time intervals, swollen beads were carefully removed from the PBS solution and the excess water blotted with filter paper from the surface. The

beads were then weighed on an analytical balance (Sartorius 2006 MP, Germany). All experiments were performed in triplicate. The following equation was used to determine the percentage degree of swelling:

$$\%\,\text{Degree of swelling} = \left[\frac{(X_w - X_d)}{X_d}\right] x100 \qquad (4)$$

where:

X_w = the mass (mg) of swollen microbeads at a predetermined time,

X_d = the mass (mg) of initial dry microbeads added to PBS solution.

4.2.1.4 TMC and ibuprofen release behaviour

TMC and ibuprofen should be in solution to be active as absorption enhancer and a therapeutic drug, respectively. Therefore, the amount of the polymer released from the dosage form should be determined parallel to the amount of drug released. Dissolution studies were performed in a six station Erweka® DT6R (Erweka® Apparatebau GmbH, Germany) dissolution apparatus fitted with a thermostat and variable speed synchronous motor, using the basket-stirring element (USP 2004:2303). Standard USP shafts and baskets were used while the standard USP flasks (glass; spherical bottom; 160 – 210 mm high; inside diameter 98 - 106 mm) were replaced with smaller versions (glass; spherical bottom; 130 – 135 mm high; inside diameter 55 - 60 mm). This was done to accommodate the use of fewer microbeads per dissolution, due to the limited amount of synthesised TMC available. The dissolution studies were performed in PBS (pH 6.8), degassed through a 0.45 µm cellulose acetate filter (Sartorius AG, Goettingen, Germany), at a temperature of 37 ± 0.5 °C (regulated by the thermostat). The basket was adjusted to rotate smoothly at 25 ± 2 mm from the bottom of the flask at a speed of 75 rpm (kept constant by the synchronous motor).

The percentage drug content of ibuprofen for each microbead formulation was used to determine the amount of beads equivalent to 50 mg ibuprofen. The % DC of TMC for each microbead formulation was then used to determine the amount of TMC administered from the weight of dry beads used per dissolution. The required beads were placed in a dry basket. The basket was lowered into 150 ml dissolution medium, the rotation started immediately after the basket was submerged in the medium and the time recorded as t=0. At times t=5, 10, 15, 30, 60, 180, 360 and 720 minutes, 5 ml were withdrawn from each vessel through a filter unit containing a glassfibre prefilter (Sartorius AG, Goettingen, Germany) and transferred to glass poly-tops. Immediately after sampling, the volume lost was replaced with an equal volume of fresh, preheated dissolution medium. Equation 5 was used during calculation of the amount dissolved at each sampling time to correct for the drug and TMC lost through sampling.

$$Y^{*}_{n} = \left(Y_{n} + \frac{V_{s}}{V_{m}} \right) x \left(\sum^{n-1} Y^{*} \right) \qquad (5)$$

where:

Y^{*}_{n} = the corrected absorbency of the n^{th} sample,

Y_{n} = the measured absorbency of the n^{th} sample,

V_{s} = the sampling volume,

V_{m} = the dissolution medium volume, and

$\sum^{n-1} Y^{*}$ = the sum of all the corrected absorbencies prior to the n^{th} sample.

Dissolution studies were performed in six-fold and both ibuprofen and TMC were analysed using the same sample and using the respective spectrophotometric analysis (§ 4.2.1.2.1 and 4.2.1.2.2). All calculations were done using Microsoft® Excel XP for Windows® (Microsoft® Corporation, Seattle, Washington, USA).

Calculation of the mean dissolution time (MDT) is a model independent method used to evaluate drug release characteristics from *in vitro* dissolutions profiles. The MDT (minutes) is the first statistical moment for the cumulative dissolution process and is defined by equation 6 (Reppas and Nicolaides, 2000:232):

$$MDT = \frac{\sum_{i=1}^{n} t_{mid} \Delta X_d}{\sum_{i=1}^{n} \Delta X_d} \quad (6)$$

where:

i = the sample number,

n = the total number of sample times,

t_{mid} = the time (min) at the midpoint between i and i – 1, and

ΔX_d = the additional mass (mg) of drug dissolved between i and i – 1.

Application of the MDT provides a more accurate drug release rate as compared to the commonly used $t_{50\%}$ approach and is determined as the sum of the individual periods of time during which a specific fraction of the total dose is released (Pillay and Fassihi, 1998:49). The mean dissolution time were calculated for each formulation in each of the dissolution studies.

4.2.1.5 Statistical analysis

A significant problem in the comparison of dissolution profiles is how to quantify the degree to which two dissolution curves do or do not compare to each other. Moore and Flanner (1996:64) developed two fit factors providing a single value to describe the "closeness" of two dissolution profiles. These fit factors directly compare the difference between the percentage drug dissolved per unit time for a test and a reference formulation. The fit factors are denoted by f_1 and f_2 and can be defined by equation 7 and 8 (Reppas and Nicolaides, 2000:241):

$$f_1 = 100\left[\frac{\sum_{i=1}^{n}|R_i - T_i|}{\sum_{i=1}^{n} R_i}\right] \quad (7)$$

$$f_2 = 50\log\left[100\left(\frac{1}{\sqrt{1+\frac{1}{n}\sum_{i=1}^{n}(R_i - T_i)^2}}\right)\right] \quad (8)$$

where:

i = the sample number,

n = the total number of sample times,

R_i = percentage dissolved of the reference profile at the i time point, and

T_i = percentage dissolved of the test profile at the i time point.

The difference factor (f_1) describes the relative error between two dissolution profiles and it approximates the percent error between two curves. A plot of fit factor f_1 against the average percent error is a linear relationship. The percent error is zero when the test and reference profiles are identical and increases proportionally with the dissimilarity between the two profiles. The similarity factor (f_2) is a logarithmic transformation of the sum-squared error of differences between the test and reference profiles over all time points. A plot of the fit factor f_2 against the average deviation between the test and reference dissolution curves can be described by an exponential curve (Moore and Flanner, 1996:66). A 10 % difference between batches of the same formulation at all sample points is generally accepted to show similarity between two dissolution profiles. Thus, according to equation 7, f_1 should be 10 at most, and from equation 8, f_2 should be at least 50 for an up to 10 % difference between two curves (Reppas and Nicolaides, 2000:243). Therefore, f_1 values less than 10 and f_2 values more than

50 indicate that significant similarities exist between the reference and the test dissolution profiles.

The dissolution profiles for the different formulations were compared to each other by means of the calculated f_1 and f_2 values. Both fit factors were calculated for each comparison, to ensure that statistical significant differences existed where both f_1 and f_2 supported the differences in the dissolution profiles. Statistical analysis of the experimental data was also done to establish the degree of differences, if any, between the formulations tested. ANOVA (analysis of variation) was applied to the data using the Tukey's Studentised Range (HSD) Test from Statistica® 6 for Windows® (Microsoft® Corporation, Seattle, Washington, USA). The results were compared with each other and a p-value of < 0.05 was tested. This indicated if statistically significant differences with a confidence interval of 95 % ($p < 0.05$) were found between the formulations studied.

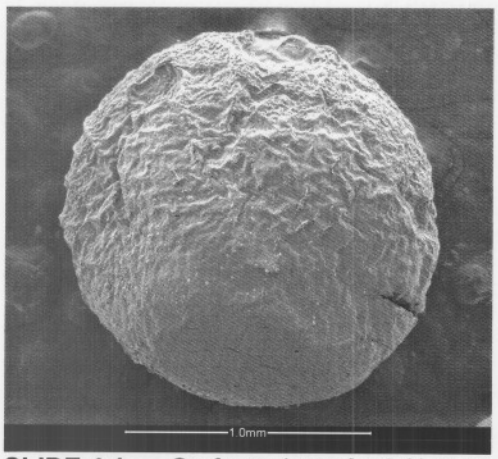
4.2.2 Results

4.2.2.1 Effect of different TMC concentrations on chitosan microbeads

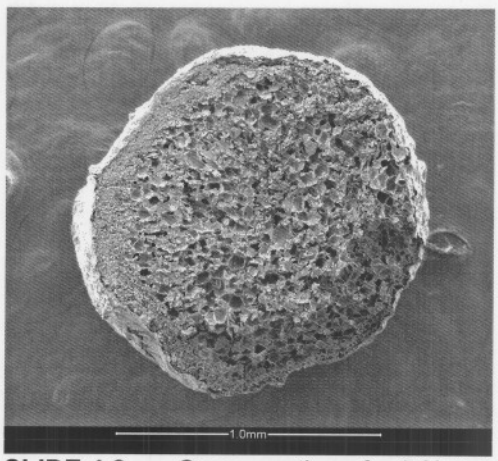
Previous studies performed by Lubbe (2002:49) showed that preparing beads by using a 3 % (w/v) chitosan solution provided more spherical beads than a 2 % chitosan solution and was also easier to prepare than using a 4 % solution due to the decreased viscosity of the 3 % (w/v) solution. Different chitosan/TMC microbeads were prepared by, keeping the chitosan concentration constant at 3 % (w/v) and varying the concentrations of either TMC-M or TMC-H between 1, 2 and 3 % (w/v). No additives or ibuprofen were added to the beads at this stage in order to study the influence of different concentrations of the TMC polymers on the morphological integrity of the chitosan microbeads.

4.2.2.1.1 Morphology

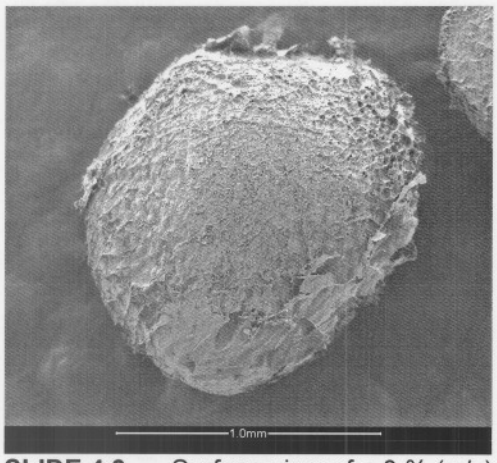
The results obtained with scanning electron microscopy (SEM) are depicted in slides 4.1 to 4.14. Microbeads prepared from 3 % (w/v) chitosan, without any TMC, were used as control for the morphological comparison of beads containing 1 to 3 % (w/v) of either TMC-M or TMC-H. The cross-sections of the beads containing TMC reveal a porous matrix network with more cavities than the control. This might be explained by the replacement of some chitosan by TMC. The incorporated TMC polymer does not exhibit crosslinking activity with TPP which might be attributed to steric hindrance from its trimethyl groups. It is noticeable from the SEM micrographs that the beads became less porous with a smoother outer-surface as the concentration of TMC increased, with TMC-H presenting denser beads compared to an equal concentration (w/v) TMC-M. The more porous beads found with the 1 % (w/v) TMC-M and TMC-H also exhibited weak structural integrity.



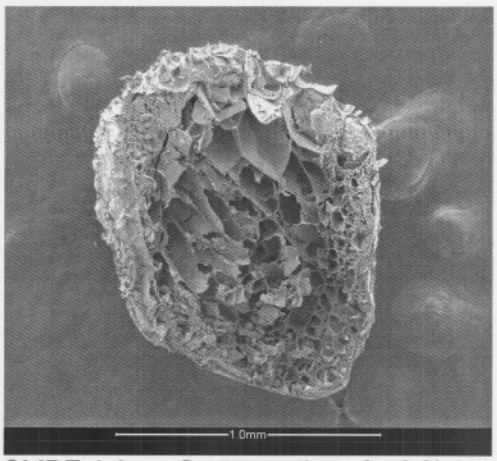
SLIDE 4.1 Surface view of a 3 % (w/v) chitosan microbead without TMC or any additives.



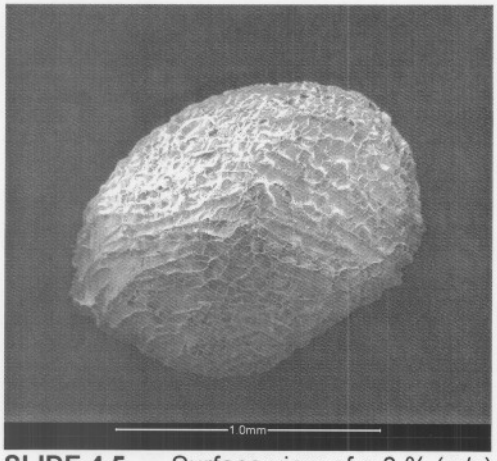
SLIDE 4.2 Cross-section of a 3 % (w/v) chitosan microbead without TMC or any additives.



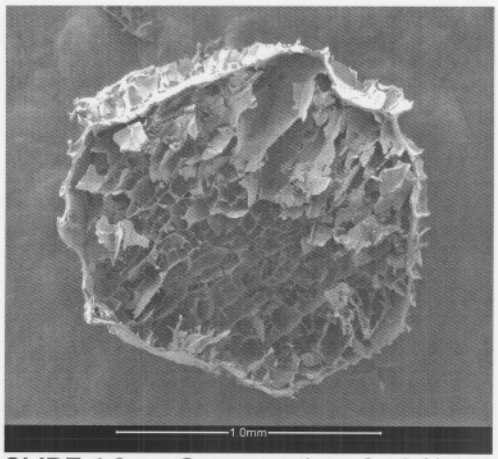
SLIDE 4.3 Surface view of a 3 % (w/v) chitosan microbead with 1 % (w/v) TMC-M included.



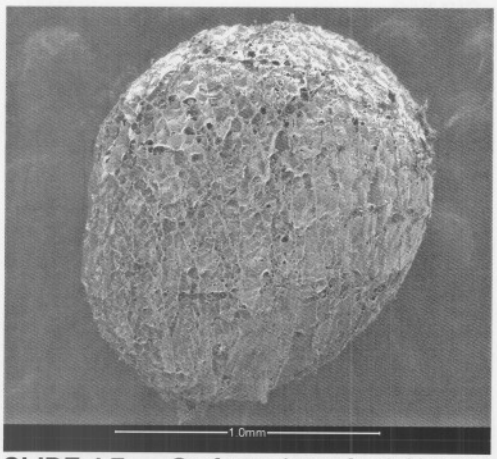
SLIDE 4.4 Cross-section of a 3 % (w/v) chitosan microbead with 1 % (w/v) TMC-M included.



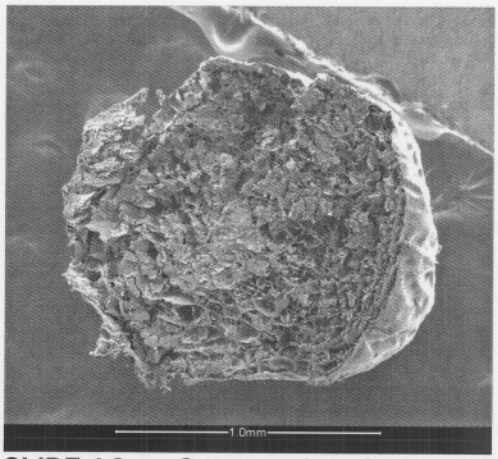
SLIDE 4.5 Surface view of a 3 % (w/v) chitosan microbead with 2 % (w/v) TMC-M included.



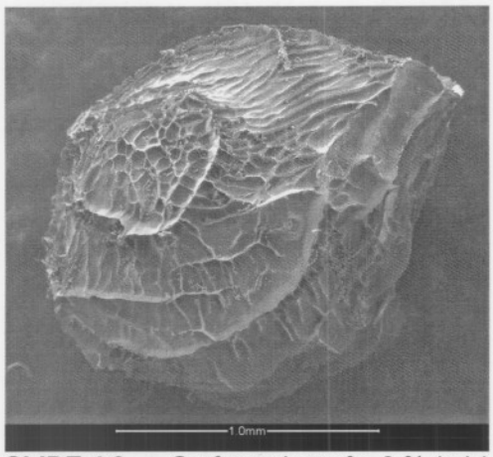
SLIDE 4.6 Cross-section of a 3 % (w/v) chitosan microbead with 2 % (w/v) TMC-M included.



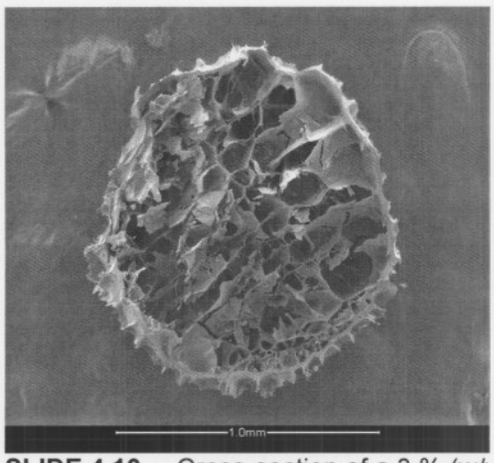
SLIDE 4.7 Surface view of a 3 % (w/v) chitosan microbead with 3 % (w/v) TMC-M included.



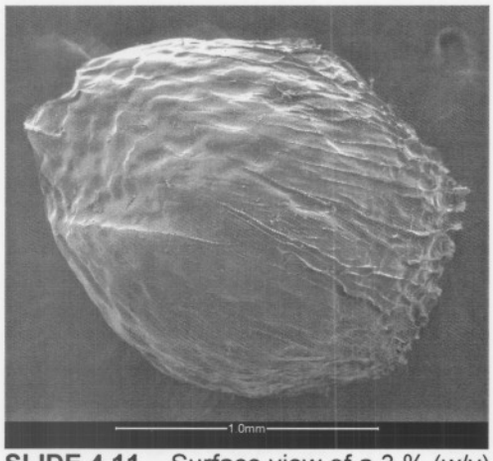
SLIDE 4.8 Cross-section of a 3 % (w/v) chitosan microbead with 3 % (w/v) TMC-M included.



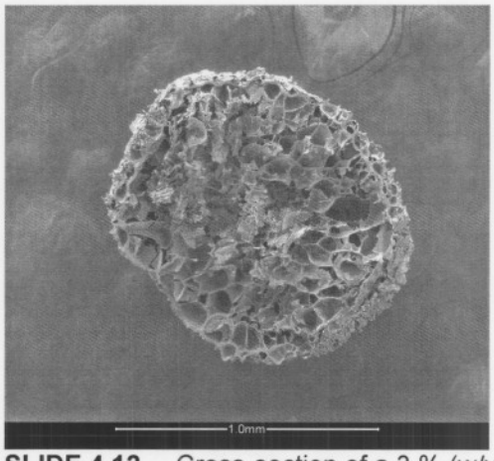
SLIDE 4.9 Surface view of a 3 % (w/v) chitosan microbead with 1 % (w/v) TMC-L included.



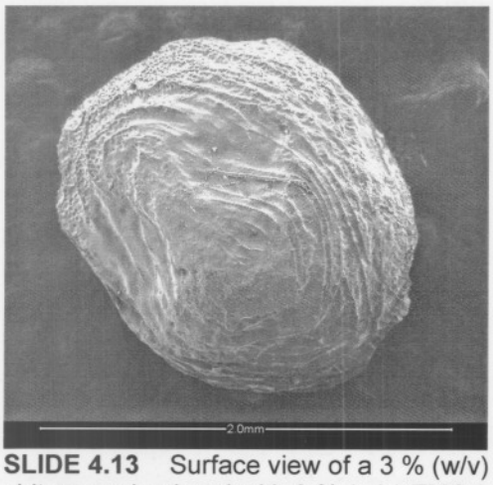
SLIDE 4.10 Cross-section of a 3 % (w/v) chitosan microbead with 1 % (w/v) TMC-L included.



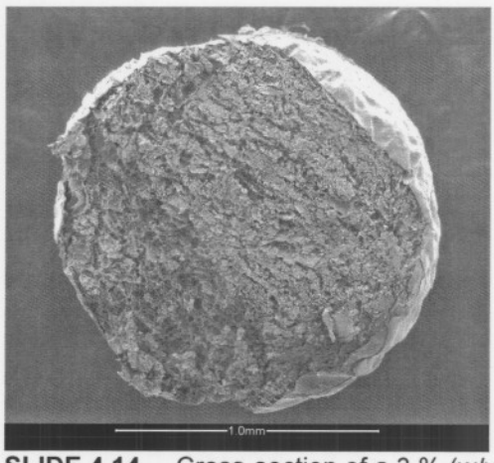
SLIDE 4.11 Surface view of a 3 % (w/v) chitosan microbead with 2 % (w/v) TMC-L included.



SLIDE 4.12 Cross-section of a 3 % (w/v) chitosan microbead with 2 % (w/v) TMC-L included.



SLIDE 4.13 Surface view of a 3 % (w/v) chitosan microbead with 3 % (w/v) TMC-L included.



SLIDE 4.14 Cross-section of a 3 % (w/v) chitosan microbead with 3 % (w/v) TMC-L included.

The denser beads prepared from 3 % TMC-M, and especially TMC-H, showed little cavities available for the possible inclusion of additives or drug molecules and also proved difficult to synthesise. Due to the high viscosity of these polymer solutions, the pressure needed to pump the solution through the needle became very high and the size of the beads increased to almost 2.0 mm.

4.2.2.1.2 TMC loading

From figure 4.3 it is clear that a straight line is obtained with standard solutions of both TMC-M and TMC-H, i.e. the Lambert-Beer law is obeyed and TMC can be determined in dilute aqueous solutions with the aid of Cibacron brilliant red 3B-A. The limit of detection for this assay was determined to be approximately 50 µg/ml. According to van der Merwe *et al.* (2004:88) it is important that samples of greater than 300 µl is taken for the analysis of TMC, otherwise a non-linear curve is obtained.

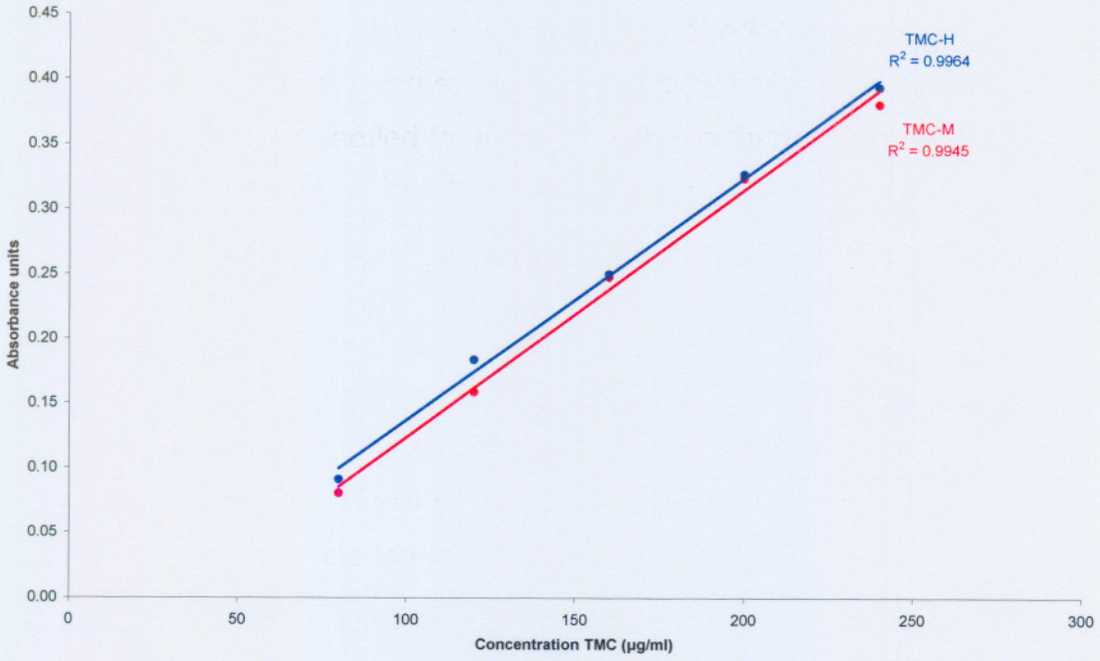


FIGURE 4.3 Standard curves for TMC-M (•) and TMC-H (•) used for the colorimetric assay of TMC.

This is attributed to the low intrinsic absorbance of TMC. By doubling the sample volume the sensitivity of the assay is increased and a linear relationship is obtained as depicted in figure 4.3. Samples of 1000 µl were used during this study for the analysis of the TMC polymers. The dry weight of the synthesised batches of microbeads varied from 1.5 to 1.7 g per batch depending on the formulation used. Higher percentage TMC added to the solution produced slightly higher dry weights. The % DLC and % DC of TMC loaded in the microbeads is depicted in table 4.2 and was determined using equations 2 and 3. It is noticed that the percentage TMC loading capacity (% DLC) and the percentage TMC content (% DC) increased for both TMC polymers with an increase of TMC added to the chitosan solution. TMC-M showed a lower loading capacity than TMC-H possibly because of poorer entanglement of the shorter TMC-M chains with the chitosan network. This resulted in TMC-M loss from the microbeads during the process of bead preparation.

TABLE 4.2 Percentage TMC loading capacity (% DLC) and TMC content (% DC) of varying concentrations (1, 2 and 3 %) TMC-M and TMC-H incorporated into chitosan microbeads.

FORMULATION	% DLC	% DC
TMC-M 0.1 %	34.0	4.0
TMC-M 0.2 %	56.0	13.5
TMC-M 0.3 %	65.0	23.0
TMC-H 0.1 %	62.0	8.0
TMC-H 0.2 %	77.0	19.0
TMC-H 0.3 %	83.0	29.0

4.2.2.1.3 Swelling behaviour studies

The percentage degree of swelling is an indication of the amount of aqueous medium absorbed and was performed in triplicate for all chitosan microbead variations at 15 minutes, 1, 3, 6, and 12 hours using equation 4. Figures 4.4 and 4.5 illustrate the % degree of swelling as a function of time for TMC-M and TMC-H respectively, with plain chitosan microbeads as control. The microbeads containing TMC, as well as the control, absorbed plenty of the PBS solution (pH 6.8) during the first 15 minutes. All formulations, except the control, seemed to decrease in weight during the period from 15 minutes to 1 hour. This might be attributed to the release of TMC from the microbead formulations. Both TMC-M and TMC-H is soluble in the aqueous PBS environment (pH 6.8) where the swelling behaviour studies were performed and might rapidly dissolve initially into the surrounding solution. The TMC polymers have much higher molecular weights than that of the water and ions inside the PBS solution and therefore the decrease in weight is observed as the TMC is released and the PBS solution is absorbed. The microbeads containing TMC seemed to absorb aqueous PBS up to 6 hours after which a plato was reached.

Figure 4.6 shows a comparison of the % degree of swelling obtained over 12 hours for the control formulation as well as the microbeads containing TMC-M and TMC-H. It is noticed that an increase in the concentration of both TMC-M and TMC-H leads to a statistical significant ($p < 0.05$) increase in PBS being absorbed by the microbeads. This might be explained by the % DC obtained for each formulation and the resultant replacement of chitosan by TMC inside the bead. As the concentration of TMC increased from 1 to 3 %, an increase in % DC was visible and therefore more TMC replaced chitosan. Since TMC is soluble at pH 6.8, it dissolves as the PBS is absorbed by the beads. Therefore, an increase in the TMC content will allow more space for the absorption of PBS as the TMC dissolves into the surrounding solution.

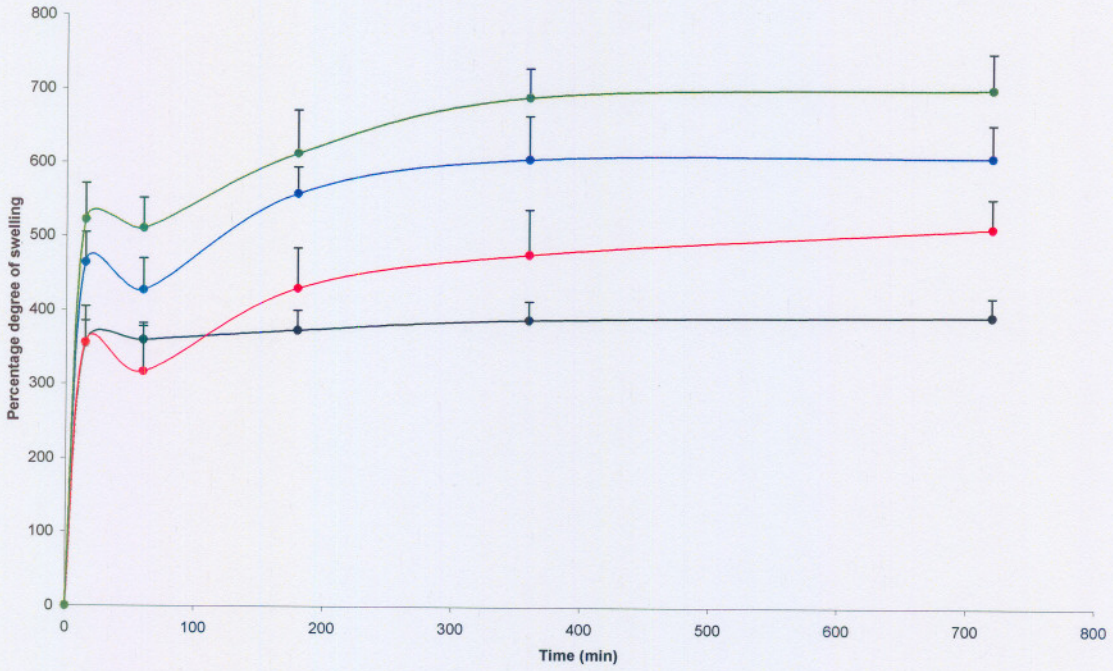


FIGURE 4.4 Percentage degree of swelling as a function of time for chitosan microbeads containing 1 % (w/v) (●), 2 % (w/v) (●) and 3 % (w/v) (●) TMC-M with plain chitosan microbeads (●) as control. Data is expressed as the mean ± SD (n=3).

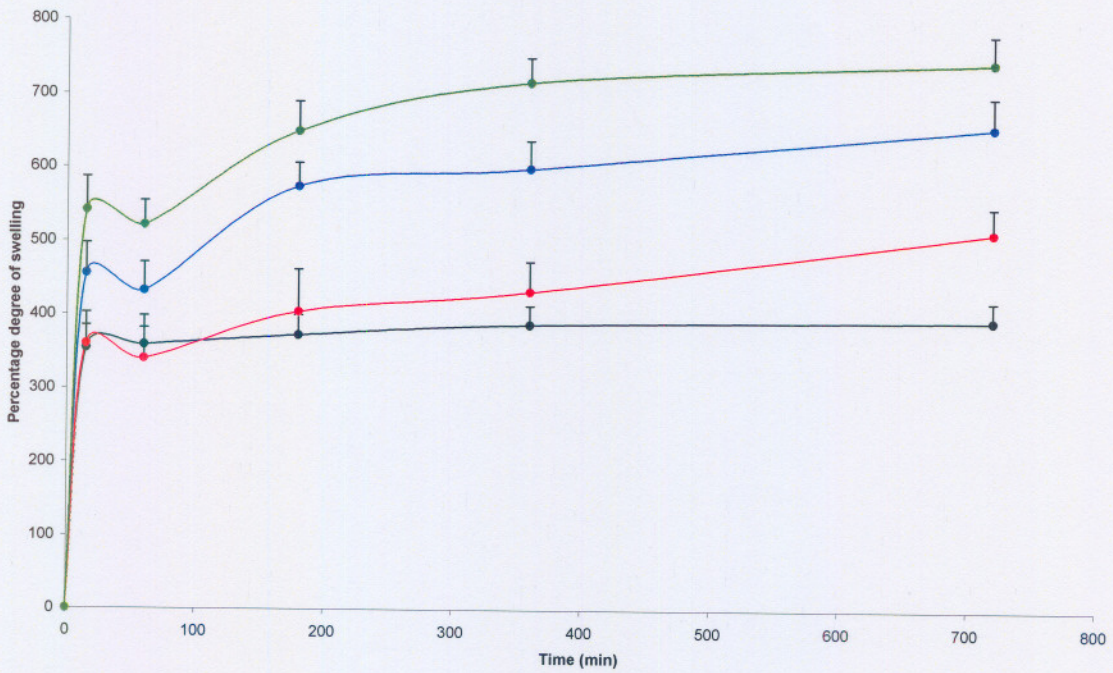


FIGURE 4.5 Percentage degree of swelling as a function of time for chitosan microbeads containing 1 % (w/v) (●), 2 % (w/v) (●) and 3 % (w/v) (●) TMC-H with plain chitosan microbeads (●) as control. Data is expressed as the mean ± SD (n=3).

This is also visible between the two distinguished TMC polymers as TMC-M exhibits lower % DC's compared to TMC-H, although no statistical significant ($p > 0.05$) differences were obtained in corresponding concentrations between the two TMC polymers. The control formula containing no TMC showed very little swelling, probably due to the strong crosslinking of chitosan by TPP and also chitosans' inability to dissolve at pH 6.8.

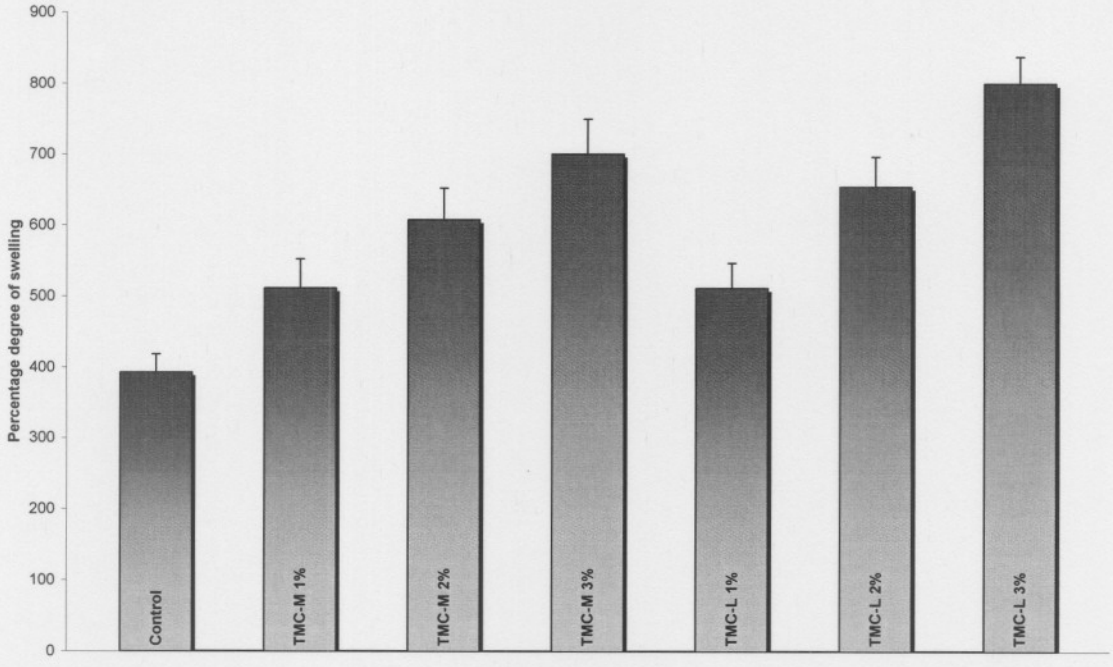


FIGURE 4.6 Percentage degree of swelling obtained at 12 hours for chitosan microbeads containing 1, 2 and 3 % (w/v) TMC-M or TMC-H with plain chitosan microbeads as control. Data is expressed as the mean ± SD (n=3).

4.2.2.1.4 Conclusion

From the results obtained thus far it was decided that a chitosan (3 % w/v) microbead containing 2 % (w/v) of either TMC-M or TMC-H will be used for further studies on the effects of different concentrations (0.1, 0.2 or 0.5 % w/v) additives (Ac-di-sol®, Explotab® or ascorbic acid). The decision was based on the following results:

- The microbeads containing 1 % (w/v) TMC were very porous and fragile, exhibited the lowest TMC loading capacity (% DLC) and did not absorb a lot of aqueous medium during swelling behaviour studies.
- The microbeads containing 2 % (w/v) TMC also exhibited porosity which might allow for the inclusion of ibuprofen and additives and were more rigid than the beads containing only 1 % (w/v) TMC. The beads further loaded an acceptable percentage of TMC (% DLC) and showed promising swelling behaviour.
- The microbeads containing 3 % (w/v) TMC had a tightly packed internal matrix which might lead to low ibuprofen loading. Although the 3 % (w/v) TMC beads contained the most TMC and exhibited the best swelling behaviour, many difficulties were encountered during the preparation of the beads due to the high viscosity of the polymer solution.

It is therefore evident that the results obtained for morphology, % DLC and swelling behaviour were the most acceptable for chitosan microbeads containing 2 % (w/v) of either TMC-M or TMC-H.

4.2.2.2 Effect of different additives on chitosan microbeads containing TMC

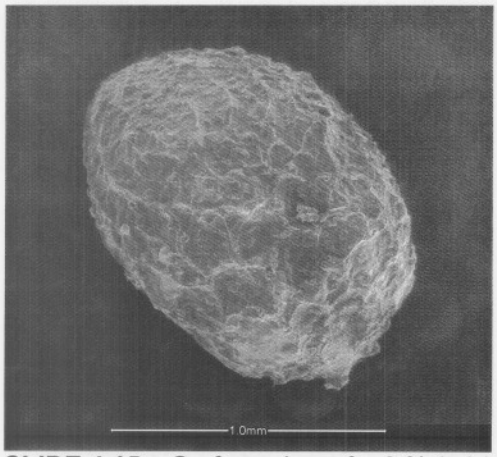
Lubbe (2002:77) indicated in previous studies that no TMC was released from freeze-dried chitosan beads (3 % w/v). Since TMC acts as a useful absorption enhancer it must be released from the beads to cause improved potential bioavailability. The addition of different excipients like Ac-di-sol®, Explotab® or ascorbic acid may improve the swelling behaviour of chitosan and therefore result in better TMC release. Different chitosan/TMC microbeads were prepared by keeping the chitosan and TMC concentration constant at 3 % (w/v) and 2 % (w/v) respectively and adding ibuprofen as model compound to all microbead formulations. Different concentrations (0.1, 0.2 and 0.5 %) of each additive were added to the microbeads in order to study the influence of additives on the release characteristics of TMC-M, TMC-H and ibuprofen.

4.2.2.2.1 Morphology

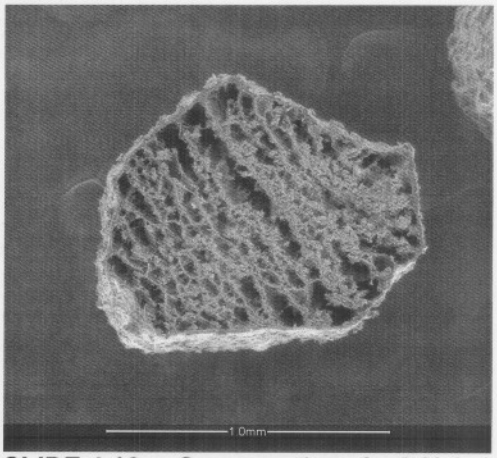
The results obtained with scanning electron microscopy (SEM) are depicted in slides 4.15 to 4.56. Microbeads containing TMC-M or TMC-H with either Ac-di-sol®, Explotab® or ascorbic acid in varying concentrations were compared with plain chitosan, chitosan containing TMC-M and chitosan containing TMC-H beads. All variants had ibuprofen included in the formulation. The morphological appearance of the synthesised microbead formulations corresponded well with each other and no significant differences were noted between the microbeads containing different TMC polymers and additives. All bead variants had a smooth outer surface with visible quantities of ibuprofen crystals entrapped in the chitosan gel structure. The dense inner matrix was found with all bead variants suggesting the loading of TMC, ibuprofen and additives into the crosslinked chitosan structure. The inclusion of different excipients in varying concentrations seemed to have little effect on the morphological integrity of the microbeads. The beads from the different formulations were also rigid and exhibited favourable mechanical strength after lyophilisation.

4.2.2.2.2 TMC and ibuprofen loading

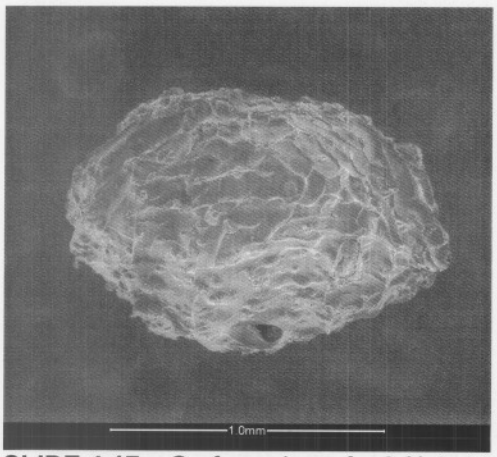
Figure 4.7 shows an example of a standard curve obtained for ibuprofen and it is clear that a straight line is also obtained with standard solutions of ibuprofen. The dry weight of the synthesised batches of microbeads again varied from 1.5 to 1.7 g per batch depending on the formulation used. Eighteen variations of chitosan microbeads (table 4.1) were synthesised and compared with (1) chitosan microbeads containing only ibuprofen (CHIT), (2) chitosan microbeads containing ibuprofen and TMC-M (CHIT-M) and (3) chitosan microbeads containing ibuprofen and TMC-H (CHIT-H) as controls. The percentage drug loading capacity (% DLC) and percentage drug content (% DC) of TMC and ibuprofen loaded in the microbeads is depicted in table 4.3 and was determined using equations 2 and 3.



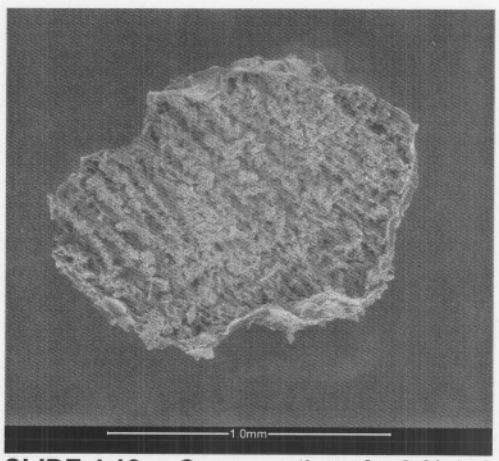
SLIDE 4.15 Surface view of a 3 % (w/v) chitosan microbead with 2 % (w/v) ibuprofen and no TMC or additives added.



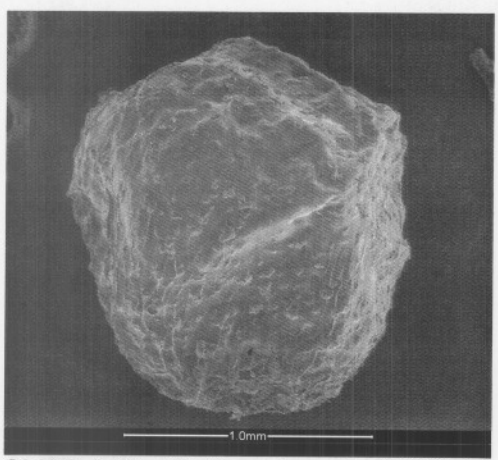
SLIDE 4.16 Cross-section of a 3 % (w/v) chitosan microbead with 2 % (w/v) ibuprofen and no TMC or additives added.



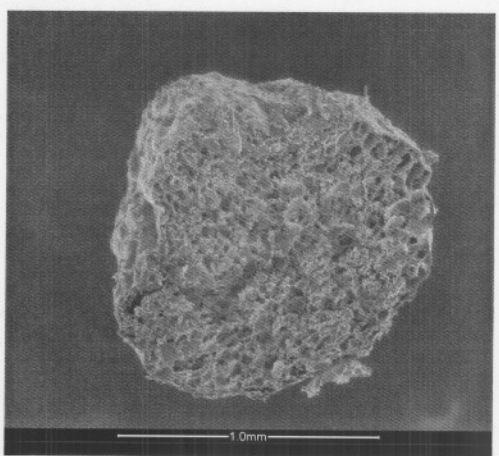
SLIDE 4.17 Surface view of a 3 % (w/v) chitosan microbead with 2 % (w/v) TMC-M and 2 % (w/v) ibuprofen included.



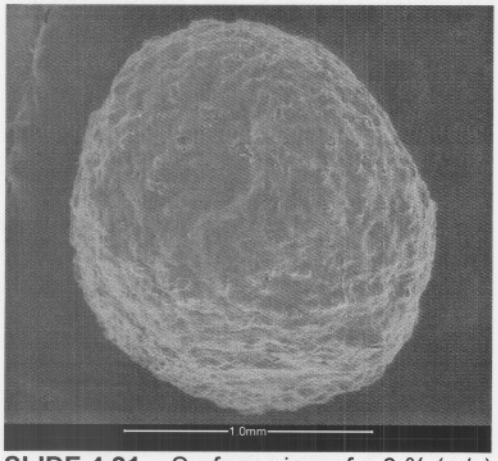
SLIDE 4.18 Cross-section of a 3 % (w/v) chitosan microbead with 2 % (w/v) TMC-M and 2 % (w/v) ibuprofen included.



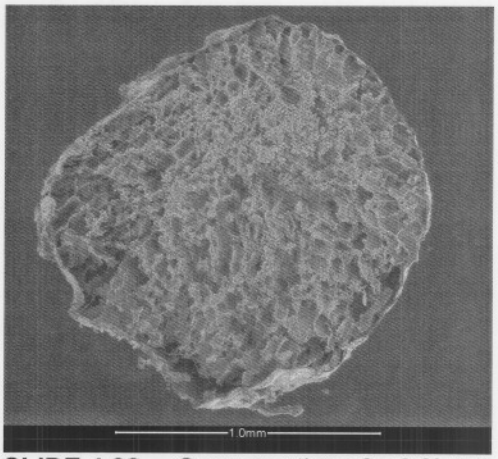
SLIDE 4.19 Surface view of a 3 % (w/v) chitosan microbead with 2 % (w/v) TMC-H and 2 % (w/v) ibuprofen included.



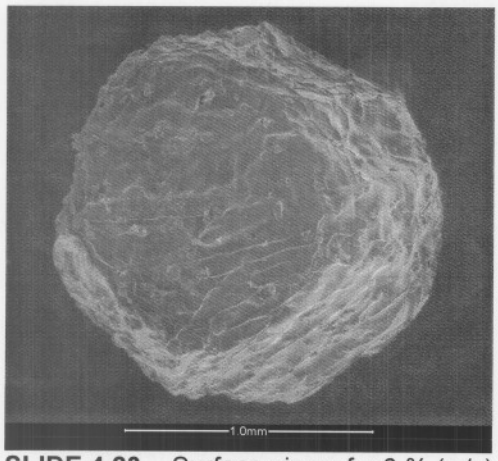
SLIDE 4.20 Cross-section of a 3 % (w/v) chitosan microbead with 2 % (w/v) TMC-H and 2 % (w/v) ibuprofen included.



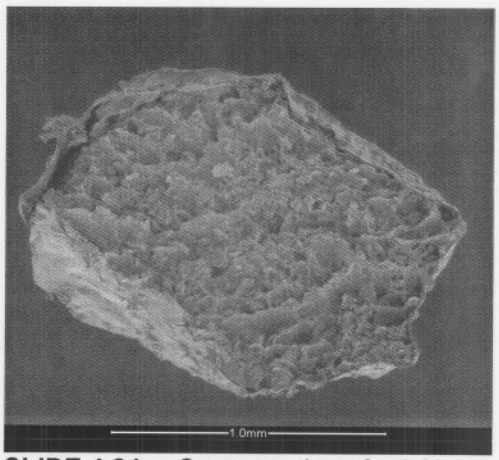
SLIDE 4.21 Surface view of a 3 % (w/v) chitosan microbead with 2 % (w/v) TMC-M, 2 % (w/v) ibuprofen, 0.1 % (w/v) Ac-di-sol®.



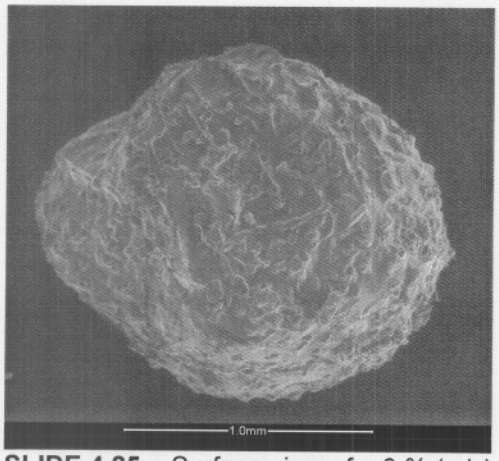
SLIDE 4.22 Cross-section of a 3 % (w/v) chitosan microbead with 2 % (w/v) TMC-M, 2 % (w/v) ibuprofen, 0.1 % (w/v) Ac-di-sol®.



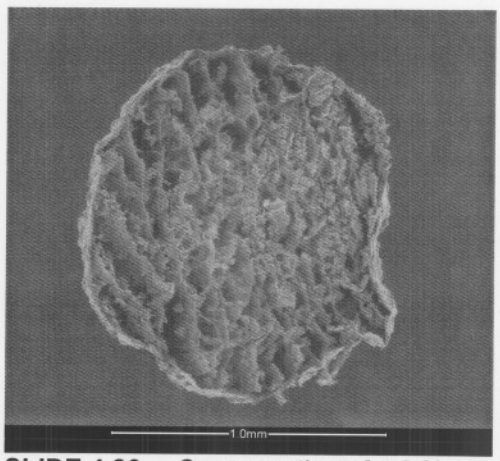
SLIDE 4.23 Surface view of a 3 % (w/v) chitosan microbead with 2 % (w/v) TMC-M, 2 % (w/v) ibuprofen, 0.2 % (w/v) Ac-di-sol®.



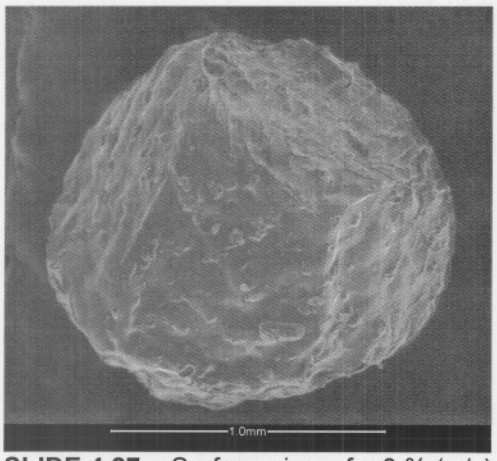
SLIDE 4.24 Cross-section of a 3 % (w/v) chitosan microbead with 2 % (w/v) TMC-M, 2 % (w/v) ibuprofen, 0.2 % (w/v) Ac-di-sol®.



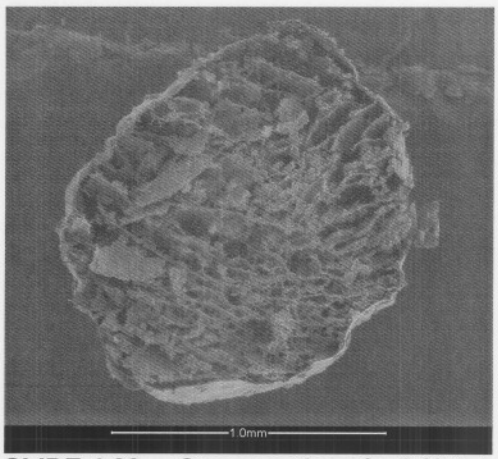
SLIDE 4.25 Surface view of a 3 % (w/v) chitosan microbead with 2 % (w/v) TMC-M, 2 % (w/v) ibuprofen, 0.5 % (w/v) Ac-di-sol®.



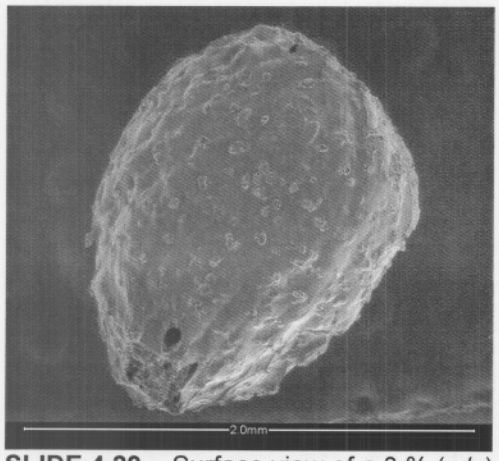
SLIDE 4.26 Cross-section of a 3 % (w/v) chitosan microbead with 2 % (w/v) TMC-M, 2 % (w/v) ibuprofen, 0.5 % (w/v) Ac-di-sol®.



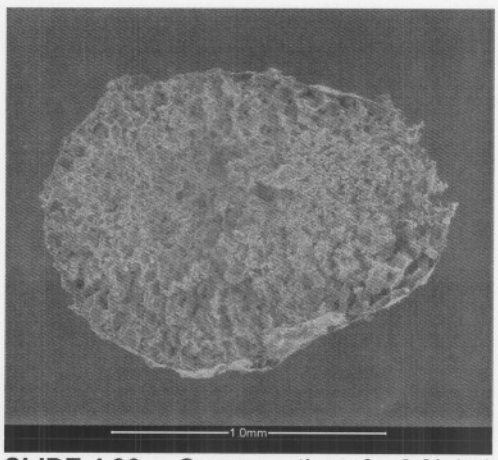
SLIDE 4.27 Surface view of a 3 % (w/v) chitosan microbead with 2 % (w/v) TMC-M, 2 % (w/v) ibuprofen, 0.1 % (w/v) Explotab®.



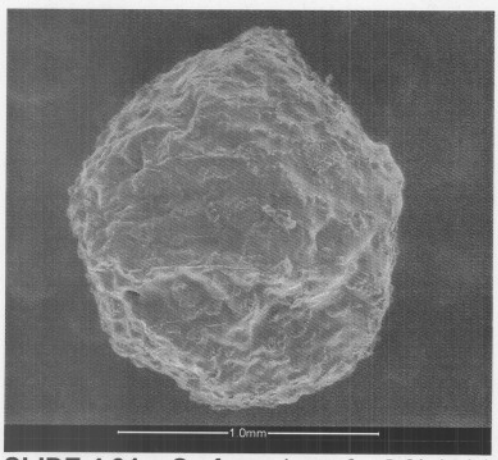
SLIDE 4.28 Cross-section of a 3 % (w/v) chitosan microbead with 2 % (w/v) TMC-M, 2 % (w/v) ibuprofen, 0.1 % (w/v) Explotab®.



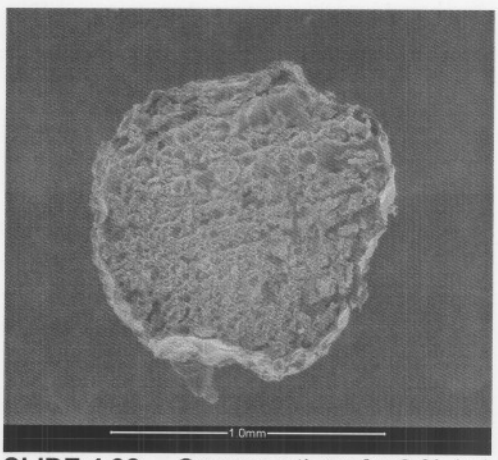
SLIDE 4.29 Surface view of a 3 % (w/v) chitosan microbead with 2 % (w/v) TMC-M, 2 % (w/v) ibuprofen, 0.2 % (w/v) Explotab®.



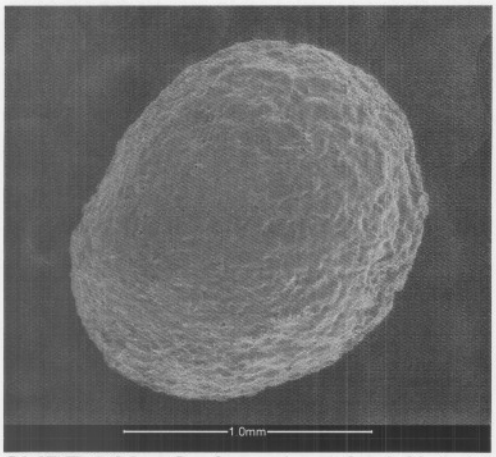
SLIDE 4.30 Cross-section of a 3 % (w/v) chitosan microbead with 2 % (w/v) TMC-M, 2 % (w/v) ibuprofen, 0.2 % (w/v) Explotab®.



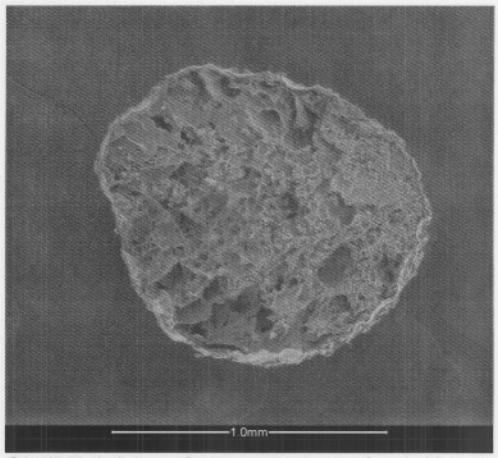
SLIDE 4.31 Surface view of a 3 % (w/v) chitosan microbead with 2 % (w/v) TMC-M, 2 % (w/v) ibuprofen, 0.5 % (w/v) Explotab®.



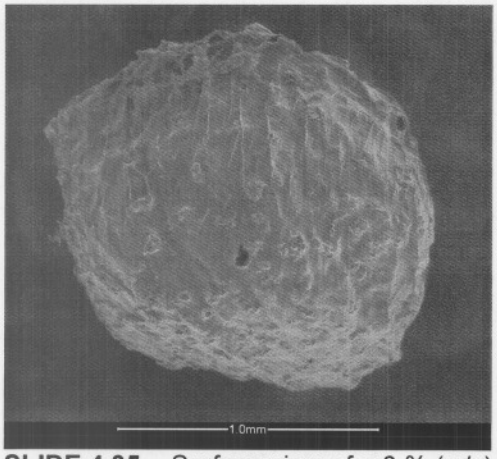
SLIDE 4.32 Cross-section of a 3 % (w/v) chitosan microbead with 2 % (w/v) TMC-M, 2 % (w/v) ibuprofen, 0.5 % (w/v) Explotab®.



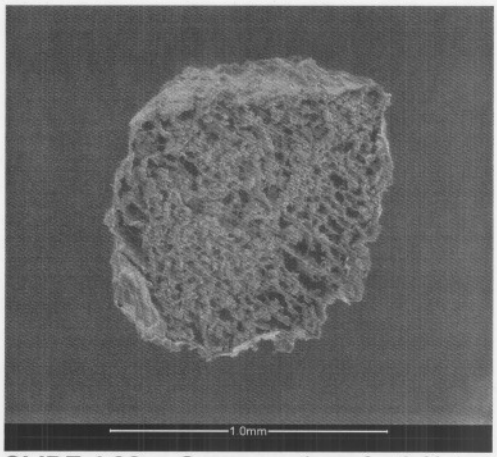
SLIDE 4.33 Surface view of a 3 % (w/v) chitosan microbead with 2 % (w/v) TMC-M, 2 % (w/v) ibuprofen, 0.1 % (w/v) ascorbic.



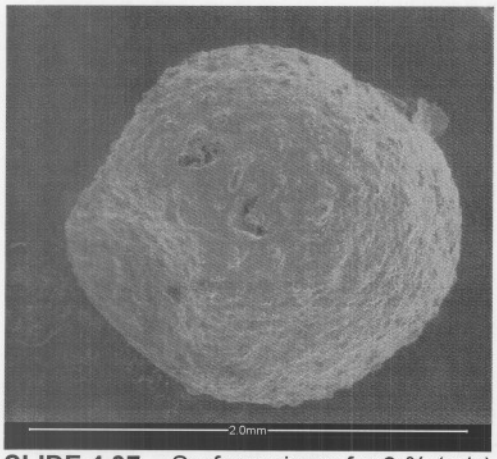
SLIDE 4.34 Cross-section of a 3 % (w/v) chitosan microbead with 2 % (w/v) TMC-M, 2 % (w/v) ibuprofen, 0.1 % (w/v) ascorbic.



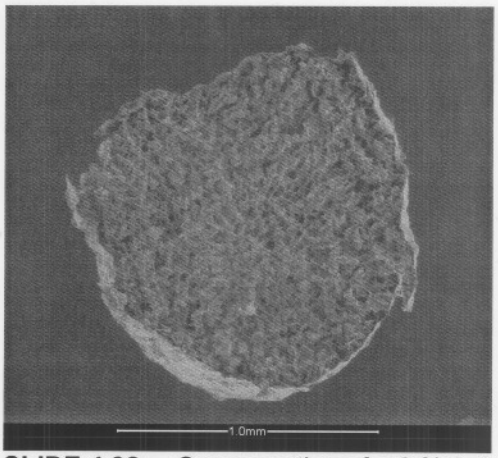
SLIDE 4.35 Surface view of a 3 % (w/v) chitosan microbead with 2 % (w/v) TMC-M, 2 % (w/v) ibuprofen, 0.2 % (w/v) ascorbic.



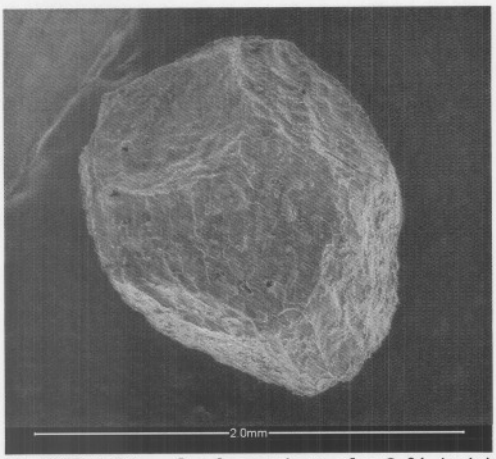
SLIDE 4.36 Cross-section of a 3 % (w/v) chitosan microbead with 2 % (w/v) TMC-M, 2 % (w/v) ibuprofen, 0.2 % (w/v) ascorbic.



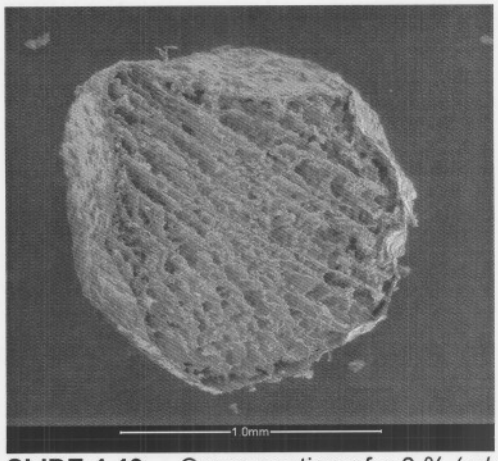
SLIDE 4.37 Surface view of a 3 % (w/v) chitosan microbead with 2 % (w/v) TMC-M, 2 % (w/v) ibuprofen, 0.5 % (w/v) ascorbic.



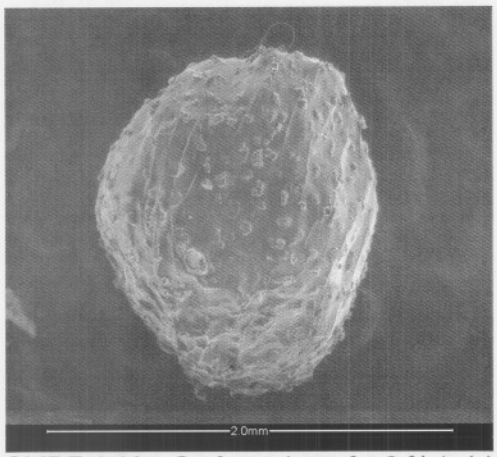
SLIDE 4.38 Cross-section of a 3 % (w/v) chitosan microbead with 2 % (w/v) TMC-M, 2 % (w/v) ibuprofen, 0.5 % (w/v) ascorbic.



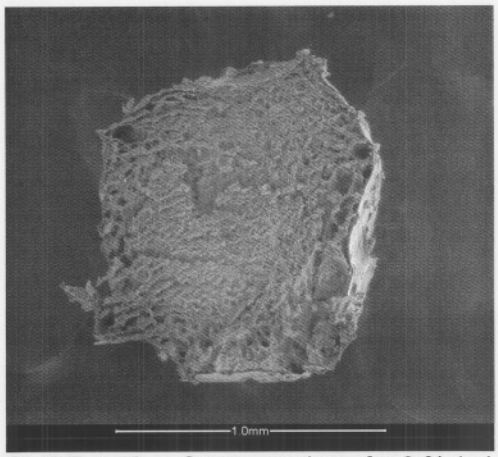
SLIDE 4.39 Surface view of a 3 % (w/v) chitosan microbead with 2 % (w/v) TMC-H, 2 % (w/v) ibuprofen, 0.1 % (w/v) Ac-di-sol®.



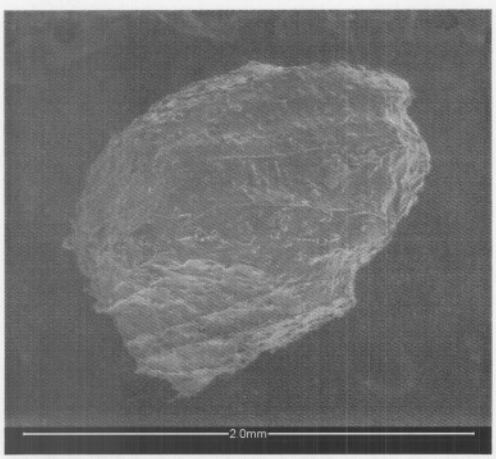
SLIDE 4.40 Cross-section of a 3 % (w/v) chitosan microbead with 2 % (w/v) TMC-H, 2 % (w/v) ibuprofen, 0.1 % (w/v) Ac-di-sol®.



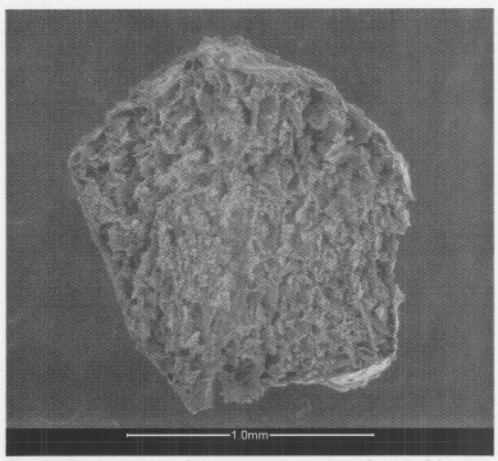
SLIDE 4.41 Surface view of a 3 % (w/v) chitosan microbead with 2 % (w/v) TMC-H, 2 % (w/v) ibuprofen, 0.2 % (w/v) Ac-di-sol®.



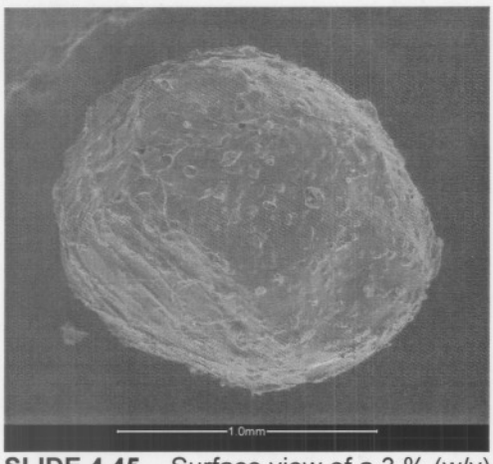
SLIDE 4.42 Cross-section of a 3 % (w/v) chitosan microbead with 2 % (w/v) TMC-H, 2 % (w/v) ibuprofen, 0.2 % (w/v) Ac-di-sol®.



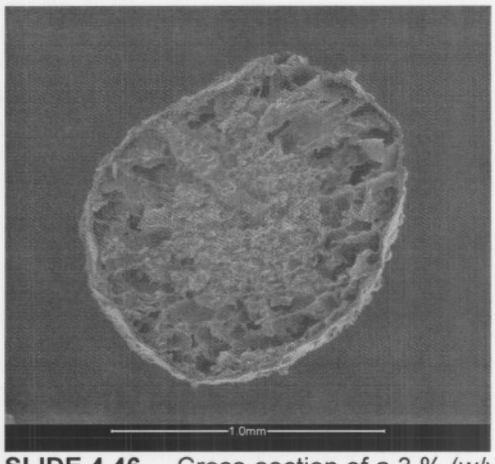
SLIDE 4.43 Surface view of a 3 % (w/v) chitosan microbead with 2 % (w/v) TMC-H, 2 % (w/v) ibuprofen, 0.5 % (w/v) Ac-di-sol®.



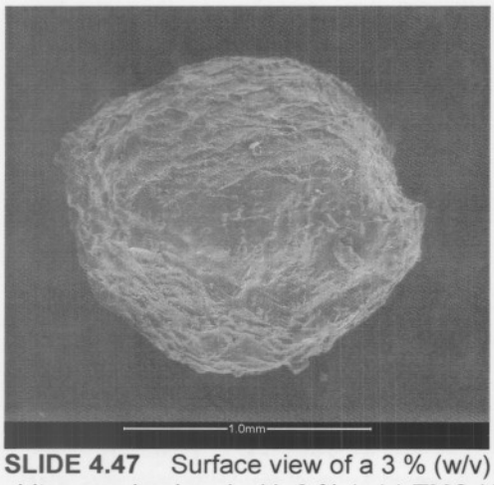
SLIDE 4.44 Cross-section of a 3 % (w/v) chitosan microbead with 2 % (w/v) TMC-H, 2 % (w/v) ibuprofen, 0.5 % (w/v) Ac-di-sol®.



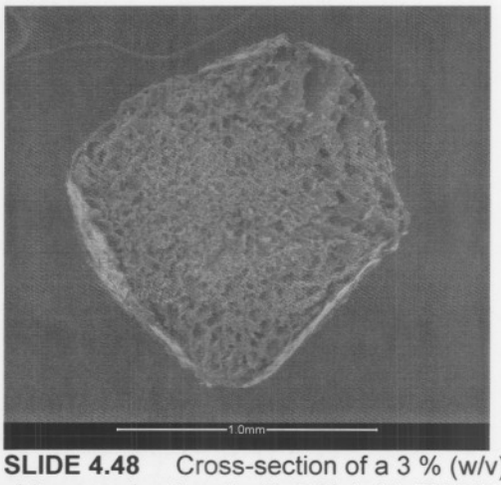
SLIDE 4.45 Surface view of a 3 % (w/v) chitosan microbead with 2 % (w/v) TMC-H, 2 % (w/v) ibuprofen, 0.1 % (w/v) Explotab®.



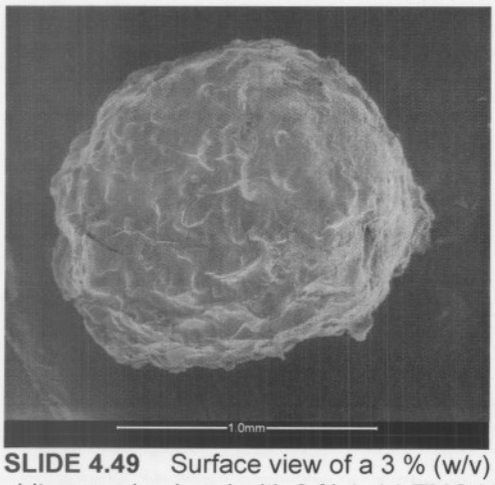
SLIDE 4.46 Cross-section of a 3 % (w/v) chitosan microbead with 2 % (w/v) TMC-H, 2 % (w/v) ibuprofen, 0.1 % (w/v) Explotab®.



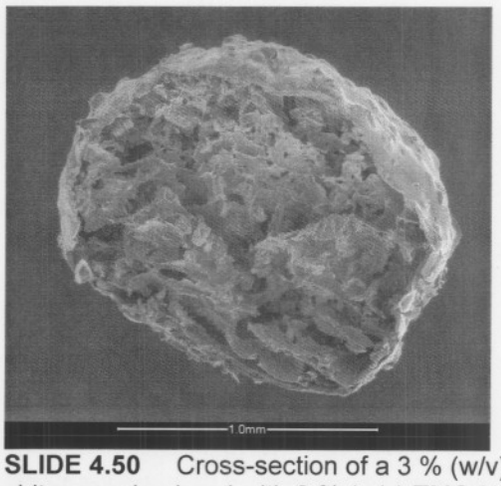
SLIDE 4.47 Surface view of a 3 % (w/v) chitosan microbead with 2 % (w/v) TMC-H, 2 % (w/v) ibuprofen, 0.2 % (w/v) Explotab®.



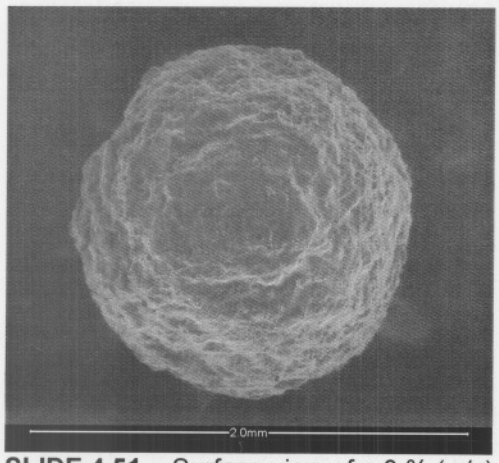
SLIDE 4.48 Cross-section of a 3 % (w/v) chitosan microbead with 2 % (w/v) TMC-H, 2 % (w/v) ibuprofen, 0.2 % (w/v) Explotab®.



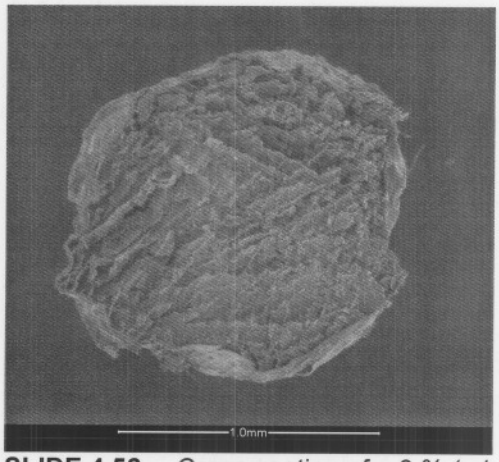
SLIDE 4.49 Surface view of a 3 % (w/v) chitosan microbead with 2 % (w/v) TMC-H, 2 % (w/v) ibuprofen, 0.5 % (w/v) Explotab®.



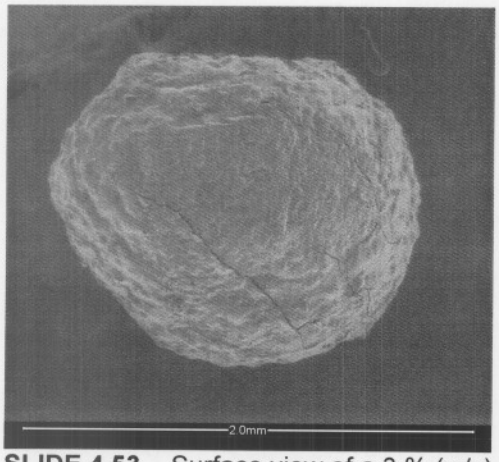
SLIDE 4.50 Cross-section of a 3 % (w/v) chitosan microbead with 2 % (w/v) TMC-H, 2 % (w/v) ibuprofen, 0.5 % (w/v) Explotab®.



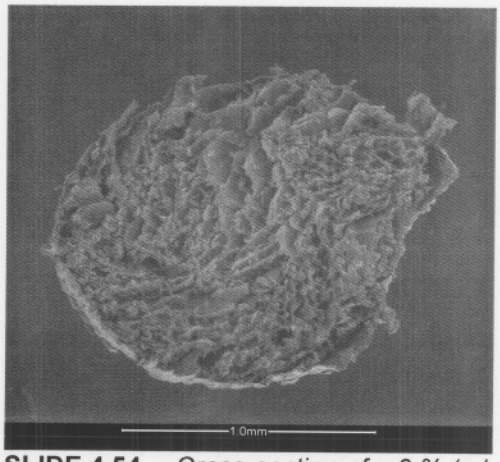
SLIDE 4.51 Surface view of a 3 % (w/v) chitosan microbead with 2 % (w/v) TMC-H, 2 % (w/v) ibuprofen, 0.1 % (w/v) ascorbic.



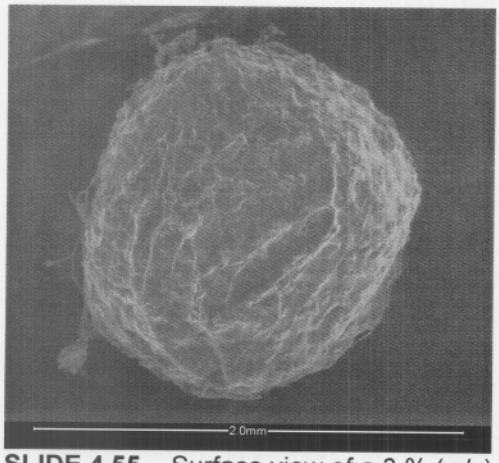
SLIDE 4.52 Cross-section of a 3 % (w/v) chitosan microbead with 2 % (w/v) TMC-H, 2 % (w/v) ibuprofen, 0.1 % (w/v) ascorbic.



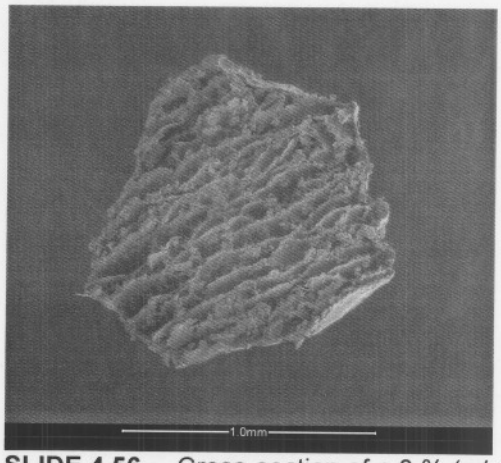
SLIDE 4.53 Surface view of a 3 % (w/v) chitosan microbead with 2 % (w/v) TMC-H, 2 % (w/v) ibuprofen, 0.2 % (w/v) ascorbic.



SLIDE 4.54 Cross-section of a 3 % (w/v) chitosan microbead with 2 % (w/v) TMC-H, 2 % (w/v) ibuprofen, 0.2 % (w/v) ascorbic.



SLIDE 4.55 Surface view of a 3 % (w/v) chitosan microbead with 2 % (w/v) TMC-H, 2 % (w/v) ibuprofen, 0.5 % (w/v) ascorbic.



SLIDE 4.56 Cross-section of a 3 % (w/v) chitosan microbead with 2 % (w/v) TMC-H, 2 % (w/v) ibuprofen, 0.5 % (w/v) ascorbic.

It is noticed that the loading (% DLC) of TMC-M is again much lower than that obtained with TMC-H, while the loading for both polymers has decreased compared to the values obtained for 2 % (w/v) TMC in table 4.2. This decrease was expected since ibuprofen and the additives filled some cavities during synthesis, which left less space for TMC loading within the porous matrix of the chitosan microbead. The inclusion of different additives in varying concentrations did not seem to have a profound influence on the loading of either TMC-M or TMC-H. The % DC of ibuprofen corresponded well between all the formulations except for the plain chitosan microbeads without any TMC or additives. The loading (% DLC) of ibuprofen was the highest in the plain chitosan microbeads since no TMC or additives competed with the ibuprofen to fill the inner cavities. Again the inclusion of different additives in varying concentrations did not seem to have a significant influence on the loading of either TMC-M or TMC-H. Although less TMC-M were loaded within the chitosan microbeads, no significant increase in ibuprofen loading was observed when compared to the beads containing TMC-H. This might indicate that the optimum % DC for ibuprofen is in the range of 18 to 22 % when TMC is present in the microbeads.

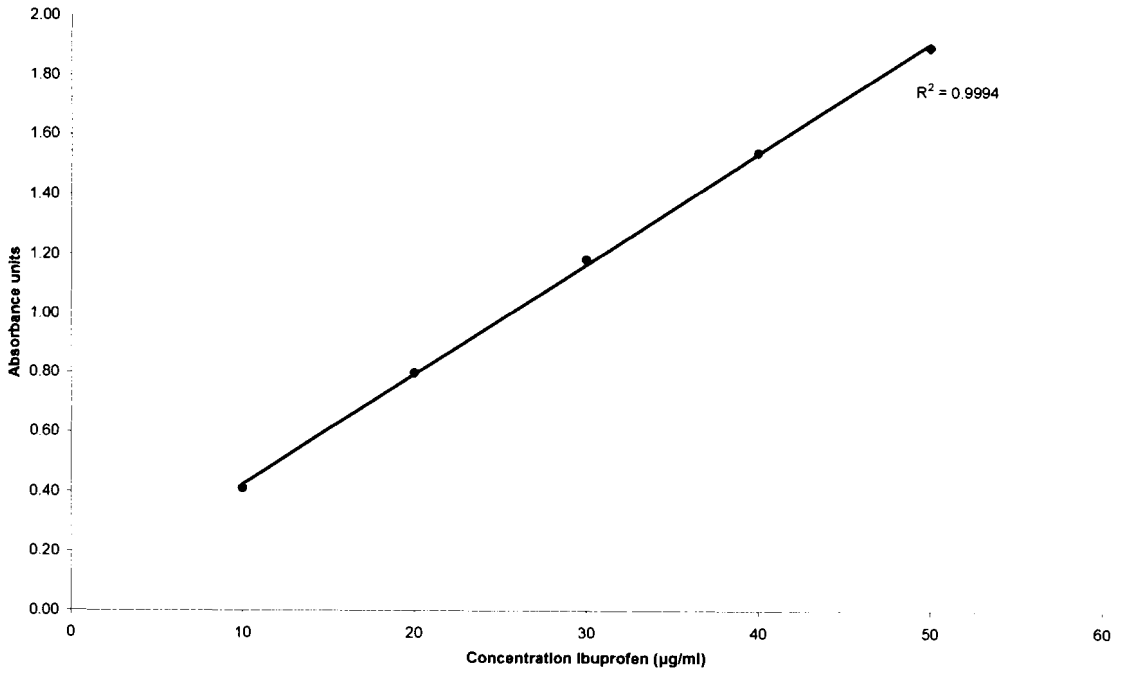


FIGURE 4.7 Standard curve used for the spectrophotometric analysis of ibuprofen.

TABLE 4.3 Percentage drug loading capacity (% DLC) and drug content (% DC) of TMC and ibuprofen obtained from varying chitosan microbead formulations with plain chitosan, TMC-M and TMC-H microbeads as controls.

FORMULATION	TMC		IBUPROFEN	
	% DLC	% DC	% DLC	% DC
CHIT	---	---	97.35	27.81
CHIT-M	15.33	4.20	74.62	20.44
M-ADS-0.1	12.57	3.99	72.21	22.92
M-ADS-0.2	32.61	7.95	73.59	17.95
M-ADS-0.5	17.56	4.95	74.62	21.02
M-EXP-0.1	20.63	5.89	73.42	20.98
M-EXP-0.2	26.55	6.90	73.64	19.13
M-EXP-0.5	18.38	5.94	73.57	20.16
M-AA-0.1	11.96	3.15	74.03	19.48
M-AA-0.2	13.18	3.61	72.64	19.90
M-AA-0.5	11.44	3.01	71.16	18.73
CHIT-H	64.64	17.47	75.79	20.48
H-ADS-0.1	68.06	16.20	75.32	17.93
H-ADS-0.2	62.61	15.85	74.57	18.88
H-ADS-0.5	66.96	15.75	75.58	17.78
H-EXP-0.1	58.13	15.71	74.18	20.05
H-EXP-0.2	54.77	14.04	75.16	19.27
H-EXP-0.5	55.67	13.92	77.56	19.39
H-AA-0.1	54.96	13.08	73.87	17.59
H-AA-0.2	56.71	12.60	70.87	15.75
H-AA-0.5	59.29	12.89	69.27	15.06

4.2.2.2.3 Swelling behaviour studies

The percentage degree of swelling was performed in triplicate for all chitosan microbead variations at 15 minutes, 1, 3, 6, and 12 hours using equation 4. Figures 4.8 to 4.13 illustrate the % degree of swelling as a function of time for the different formulations with CHIT-M, CHIT-H and plain chitosan (CHIT) as controls. All chitosan microbead formulations, as well as the controls, absorbed plenty of the PBS (pH 6.8) solution during the first 15 minutes, whereafter a decrease in weight is noticed for the following 45 minutes. The plain chitosan controls' steady decrease in weight continued for the total period of 12 hours. This might be due to the gradual and slow release of ibuprofen from the chitosan microbeads while little PBS (pH 6.8) is absorbed into the beads since no TMC is present.

The inclusion of all of the additives resulted in an increased percentage degree of swelling when compared to the controls CHIT, CHIT-M and CHIT-H. The formulations containing TMC showed an increase in weight for the period from 1 to 6 hours probably due to the majority of TMC and ibuprofen being released within the first hour. The swelling of the different microbead formulations containing TMC decreased for the period from 6 to 12 hours when compared with the period from 3 to 6 hours which suggests a saturation effect.

Figure 4.14 shows a comparison of the % degree of swelling obtained at 12 hours for the different formulations with plain chitosan microbeads as control. All the formulations containing TMC swelled significantly more than the control, while no statistical significant ($p > 0.05$) differences were obtained in the percentage degree of swelling between the different formulations.

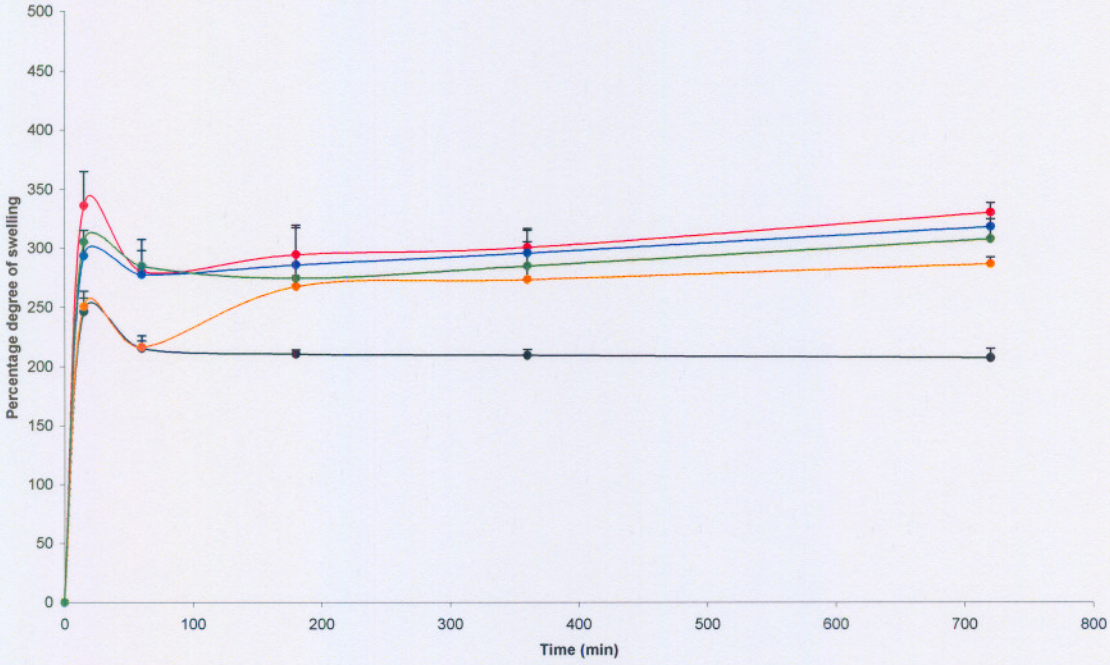


FIGURE 4.8 Percentage degree of swelling as a function of time for chitosan microbeads containing TMC-M and 0.1 % (w/v) (●), 0.2 % (w/v) (●) and 0.5 % (w/v) (●) ADS with chitosan containing TMC-M (●) and plain chitosan microbeads (●) as control. Data is expressed as the mean ± SD (n=3).

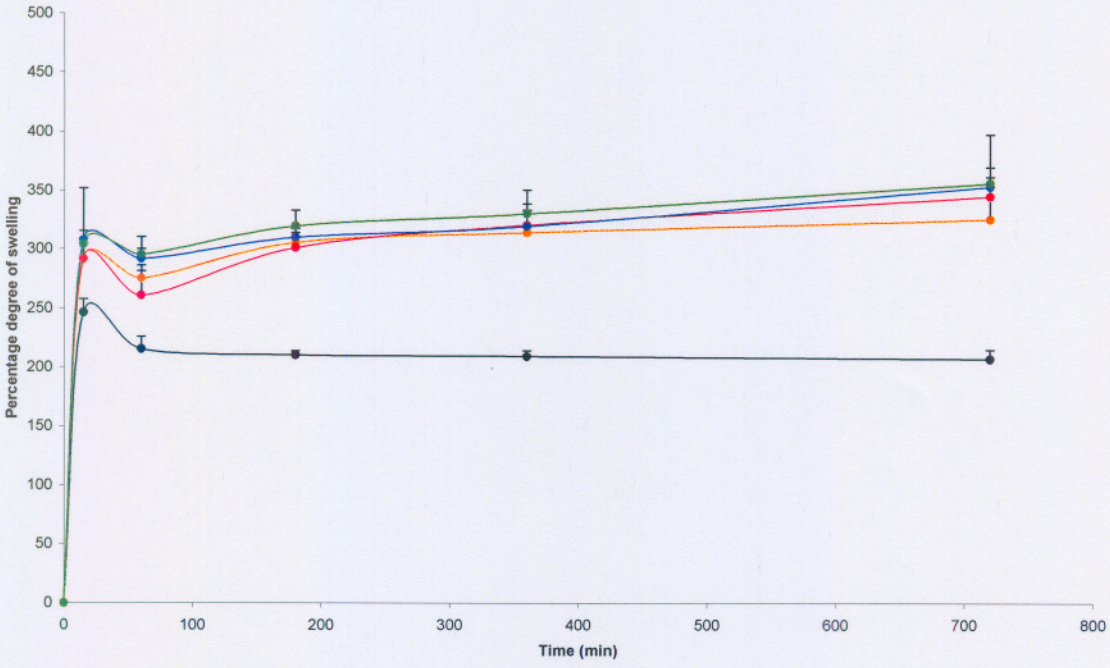


FIGURE 4.9 Percentage degree of swelling as a function of time for chitosan microbeads containing TMC-H and 0.1 % (w/v) (●), 0.2 % (w/v) (●) and 0.5 % (w/v) (●) ADS with chitosan containing TMC-H (●) and plain chitosan microbeads (●) as control. Data is expressed as the mean ± SD (n=3).

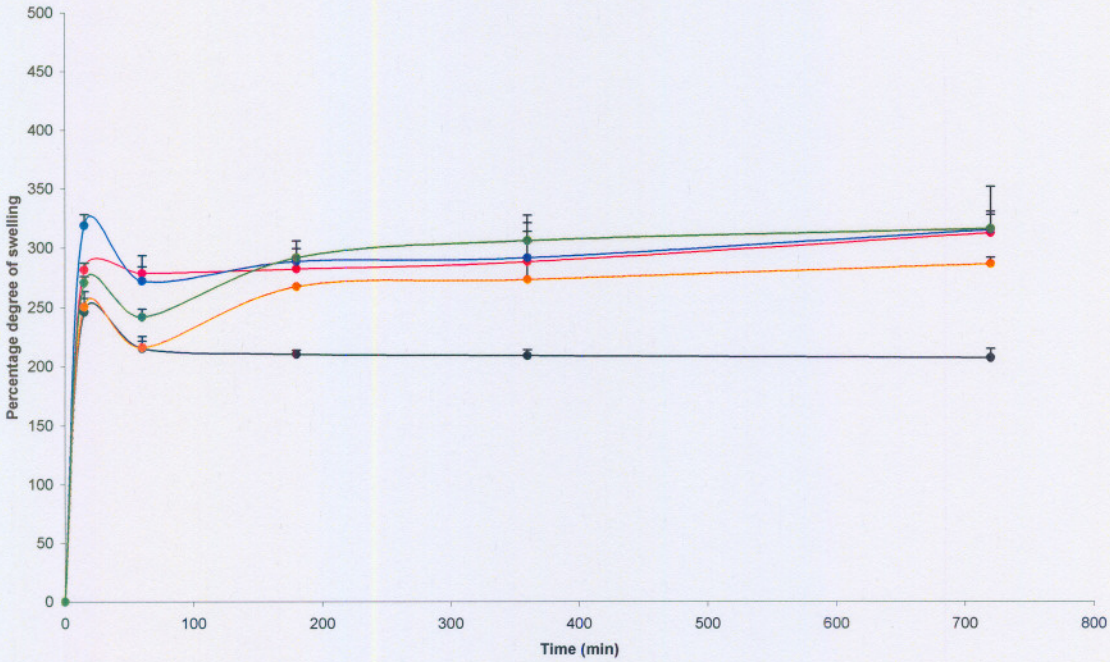


FIGURE 4.10 Percentage degree of swelling as a function of time for chitosan microbeads containing TMC-M and 0.1 % (w/v) (●), 0.2 % (w/v) (●) and 0.5 % (w/v) (●) EXP with chitosan containing TMC-M (●) and plain chitosan microbeads (●) as control. Data is expressed as the mean ± SD (n=3).

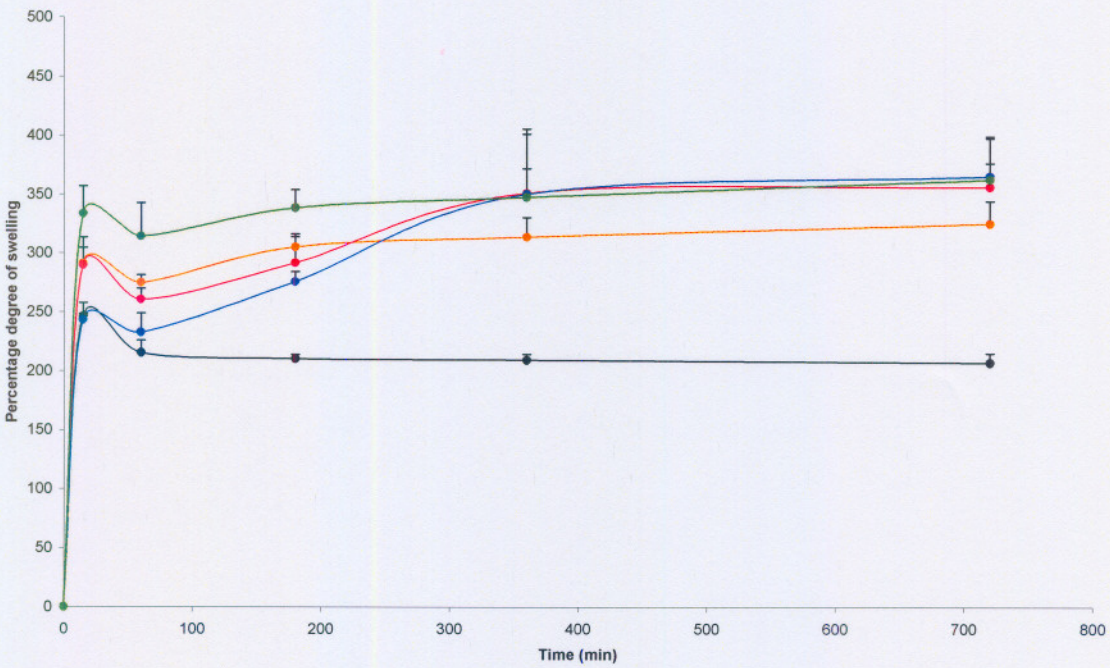


FIGURE 4.11 Percentage degree of swelling as a function of time for chitosan microbeads containing TMC-H and 0.1 % (w/v) (●), 0.2 % (w/v) (●) and 0.5 % (w/v) (●) EXP with chitosan containing TMC-H (●) and plain chitosan microbeads (●) as control. Data is expressed as the mean ± SD (n=3).

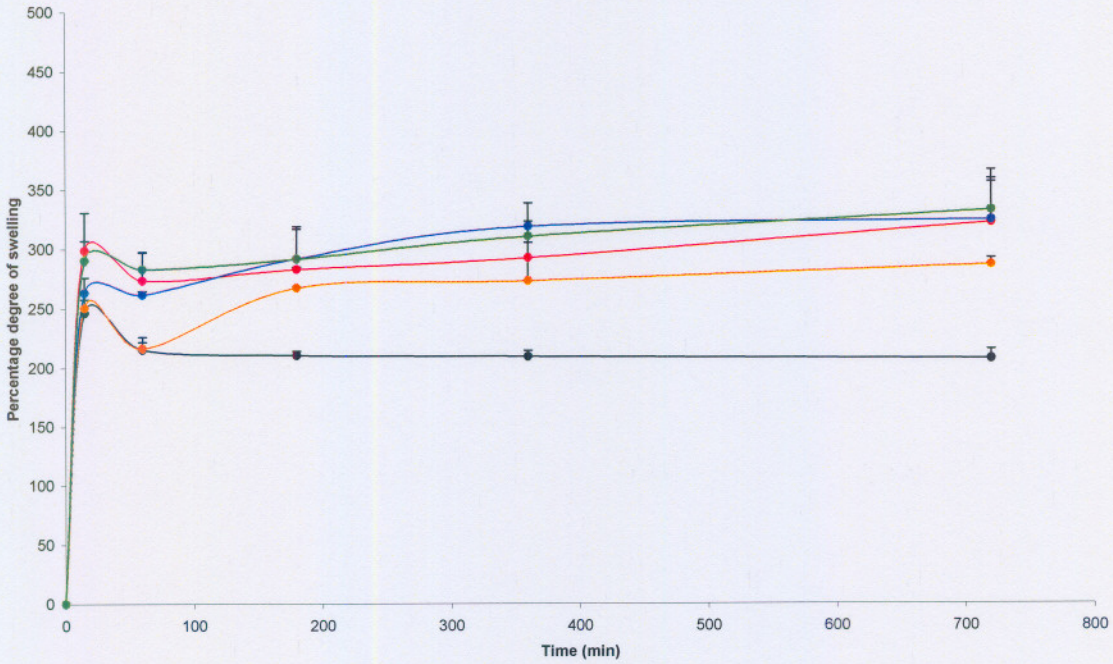


FIGURE 4.12 Percentage degree of swelling as a function of time for chitosan microbeads containing TMC-M and 0.1 % (w/v) (●), 0.2 % (w/v) (●) and 0.5 % (w/v) (●) AA with chitosan containing TMC-M (●) and plain chitosan microbeads (●) as control. Data is expressed as the mean ± SD (n=3).

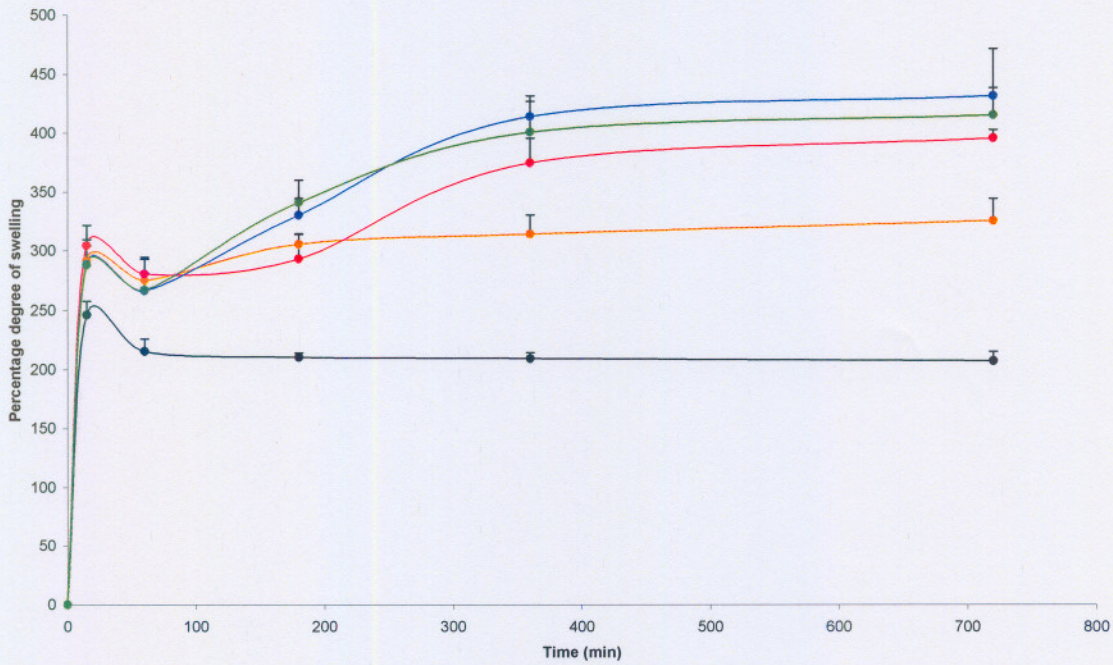


FIGURE 4.13 Percentage degree of swelling as a function of time for chitosan microbeads containing TMC-H and 0.1 % (w/v) (●), 0.2 % (w/v) (●) and 0.5 % (w/v) (●) AA with chitosan containing TMC-H (●) and plain chitosan microbeads (●) as control. Data is expressed as the mean ± SD (n=3).

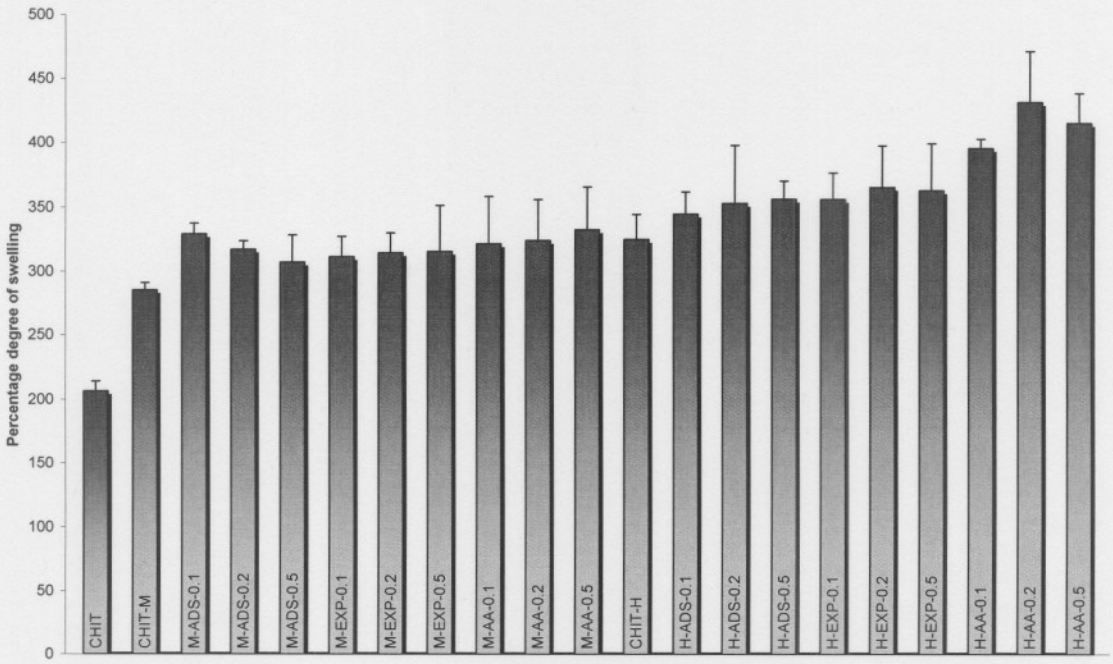


FIGURE 4.14 Percentage degree of swelling obtained at 12 hours for chitosan microbeads containing TMC-M, TMC-H and different concentrations additives with plain chitosan microbeads as control. Data is expressed as the mean ± SD (n=3).

4.2.2.2.4 TMC and ibuprofen release behaviour

The dissolution studies on all microbead formulations were performed in six-fold with CHIT, CHIT-M and CHIT-H as controls. The dissolution profiles of TMC-H and ibuprofen from the chitosan microbeads are presented in figures 4.15 to 4.17 and samples were collected at 5, 10, 15, 30, 60, 180, 360 and 720 minutes. There were no erosion of the beads observed during the dissolution studies.

TMC release

The TMC released from the microbeads containing TMC-M was below the limit of detection for the TMC analysis used and therefore no dissolution profiles are shown for the release of TMC-M. This was probably due to the poor % DC's obtained with the microbeads containing TMC-M as shown in table 4.3.

Figure 4.15 shows rapid TMC-H release from all formulations during the first 60 minutes. The high solubility of TMC-H in the aqueous medium at pH 6.8, as well as the non-crosslinking of the polymer, might be the reasons for this release. Additionally, some degree of swelling of the beads might also add to this effect. It is also noted from figure 4.15 that the formulations containing ascorbic acid, especially 0.5 % w/v, showed a statistical significant ($p< 0.05$) increase in the release of TMC-H after 12 hours when compared to those formulations containing Ac-di-sol® or Explotab®. This might be due to the increased acidity of the micro-environment within the beads, which leads to increased solubility of both chitosan and TMC-H. From 3 hours onwards an almost constant TMC-H release were obtained from all formulations for the remainder of the dissolution study (12 hours). No significant differences in the release of TMC-H obtained from the formulations containing different concentrations Ac-di-sol® or Explotab® when compared with CHIT-H as control after 12 hours was observed.

The f_1 and f_2 fit factors for the dissolution profiles of the formulations containing TMC-H are presented in tables 4.4 and 4.5 respectively. The TMC released from all the formulations containing disintegrating agents was significantly higher than the release found from CHIT-H. From tables 4.4 and 4.5 it is further evident that no significant differences between the dissolution profiles of the different concentrations of either Explotab® or Ac-di-sol® were obtained (yellow cells). H-EXP-0.5 is also similar to H–ADS-0.1 and H-ADS-0.2 with respect to TMC-H release profile and no significant difference were found between H-AA-0.1 and H-AA-0.2 (yellow cells). Comparison of all other formulations showed significant differences in their TMC-H dissolution profile (clear cells), while H-AA-0.5 were the only formulation with a significantly increased TMC-H release profile compared to all other formulations tested (blue cells).

The mean dissolution times for the formulations containing TMC-H are shown in table 4.6. The MDT of H-AA-0.5 was significantly faster compared to the MDT from CHIT-H as control. The fastest release of TMC occurred from the formulations containing ascorbic acid, with 0.5 % w/v releasing TMC-H the

fastest of all the formulations tested (MDT = 15.45 ± 1.55 minutes). Rapid release of high quantities TMC as absorption enhancer from the beads is ideal for this drug delivery system.

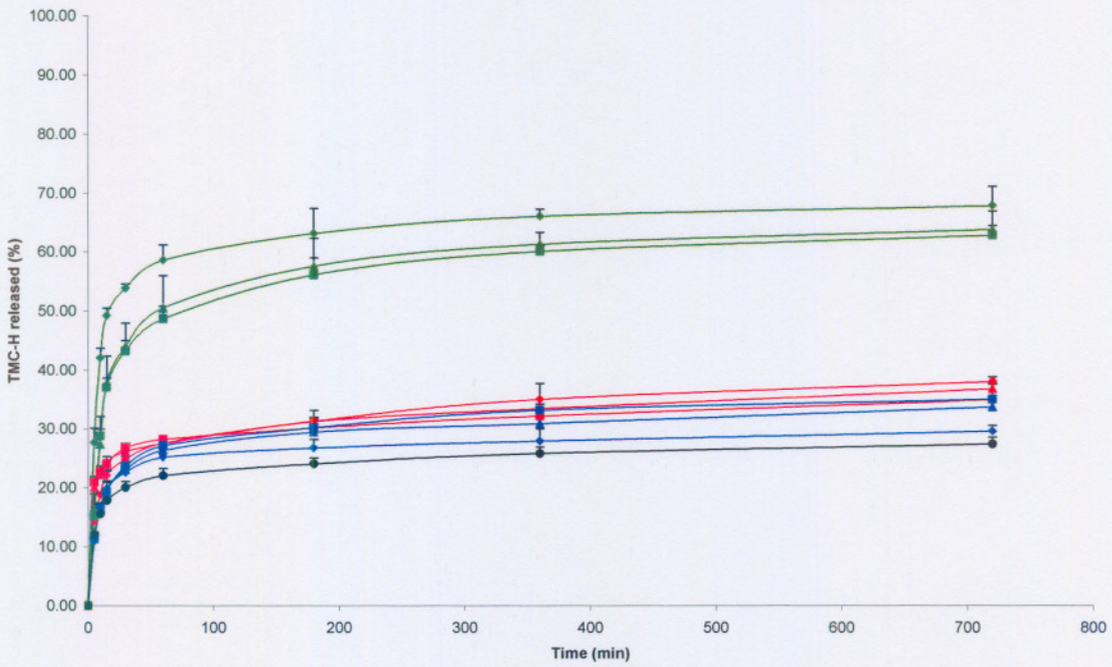


FIGURE 4.15 Dissolution profiles of TMC-H from different chitosan formulations. Legend: CHIT-H (●), H-ADS-0.1 (■), H-ADS-0.2 (▲), H-ADS-0.5 (♦), H-EXP-0.1 (■), H-EXP-0.2 (▲), H-EXP-0.5 (♦), H-AA-0.1 (■), H-AA-0.2 (▲) and H-AA-0.5 (♦). Data is expressed as the mean ± SD (n=6).

Ibuprofen release

Figures 4.16 and 4.17 illustrate the release profiles of ibuprofen from beads containing TMC-M and TMC-H respectively. A burst release effect is obtained within the first hour of the study in all formulations. This might be attributed to the solubility of ibuprofen at pH 6.8 as discussed in chapter 2. The drug was also not chemically attached to the polymer and there were only electrostatic attractions, as well as entrapment, within the polymer matrix. The maximum release of ibuprofen from the formulations containing TMC-M showed no significant differences ($p > 0.05$) from the controls and was overall less than that obtained

from the beads containing TMC-H. This might be as a result of the decreased swelling obtained with the beads containing TMC-M, in comparison with the beads containing TMC-H. The microbeads containing TMC-H released significantly more ibuprofen during the dissolution study in comparison with the controls, while the formulation containing TMC-H and 0.5 % (w/v) ascorbic acid released almost 100 % of the ibuprofen over a period of 12 hours.

The f_1 and f_2 fit factors for the dissolution profiles of all the formulations tested are presented in tables 4.4 and 4.5 respectively. Although calculation of the f_2 fit factor indicated that no significant difference existed between H-AA-0.5 in comparison with H-AA-0.2 and H-AA-0.1 (red font), the f_1 calculation did not support this finding. Therefore, it is again noticed that the formulation containing TMC-H and 0.5 % (w/v) ascorbic acid is the only formulation with a significantly higher ibuprofen release profile compared to all other formulations tested.

Table 4.6 presents the mean dissolution times for the dissolution profiles of all the formulations tested. A burst release effect of ibuprofen is observed for all the chitosan microbead formulations as indicated by the short MDT's obtained. This might be attributed to the high solubility of ibuprofen in aqueous solutions at pH 6.8. The fastest release of ibuprofen again occurred from the formulations containing ascorbic acid, with H-AA-0.5 releasing ibuprofen the fastest of all the formulations tested (MDT = 7.15 ± 0.56 minutes). Although the burst release obtained is not ideal for chitosan microbead drug delivery systems, the effect is acceptable for the purpose of this study. The rapid release of the model drug compound favours the *in vitro* permeation studies to be performed in chapter 5, since the time constraint on the experimental model is 2 hours (due to membrane degeneration). Since the ibuprofen is practically immediately available, it might allow TMC enough time to enhance the transport of the drug through biological membranes.

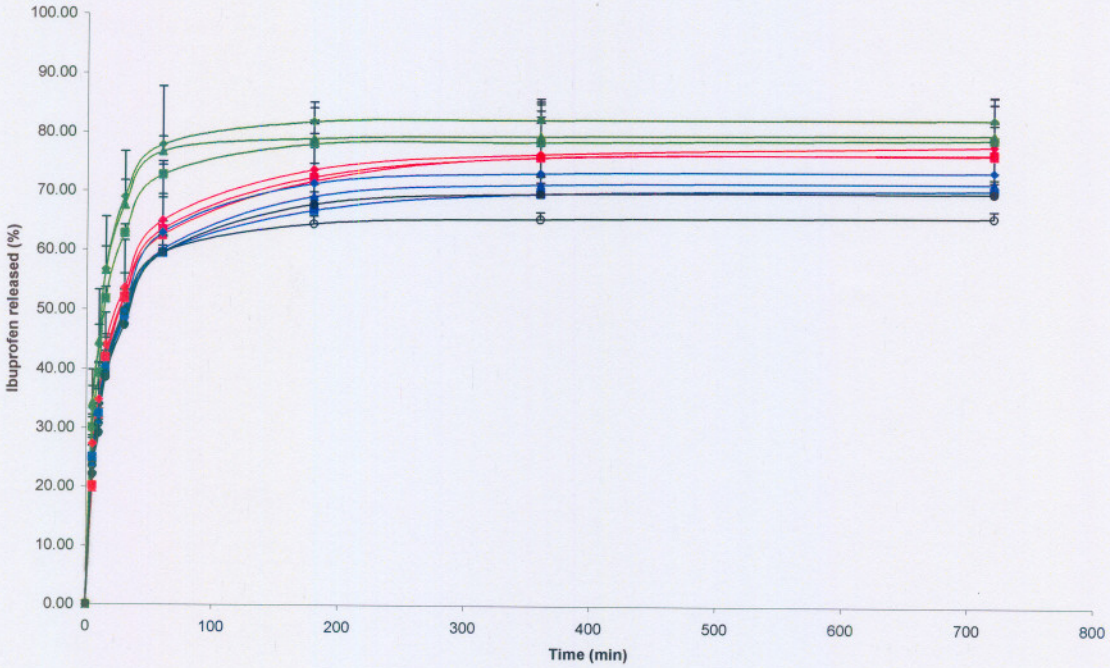


FIGURE 4.16 Dissolution profiles of ibuprofen from different TMC-M formulations. Legend: CHIT (○), CHIT-M (●), M-ADS-0.1 (■), M-ADS-0.2 (▲), M-ADS-0.5 (♦), M-EXP-0.1 (■), M-EXP-0.2 (▲), M-EXP-0.5 (♦), M-AA-0.1 (■), M-AA-0.2 (▲) and M-AA-0.5 (♦). Data is expressed as the mean ± SD (n=6).

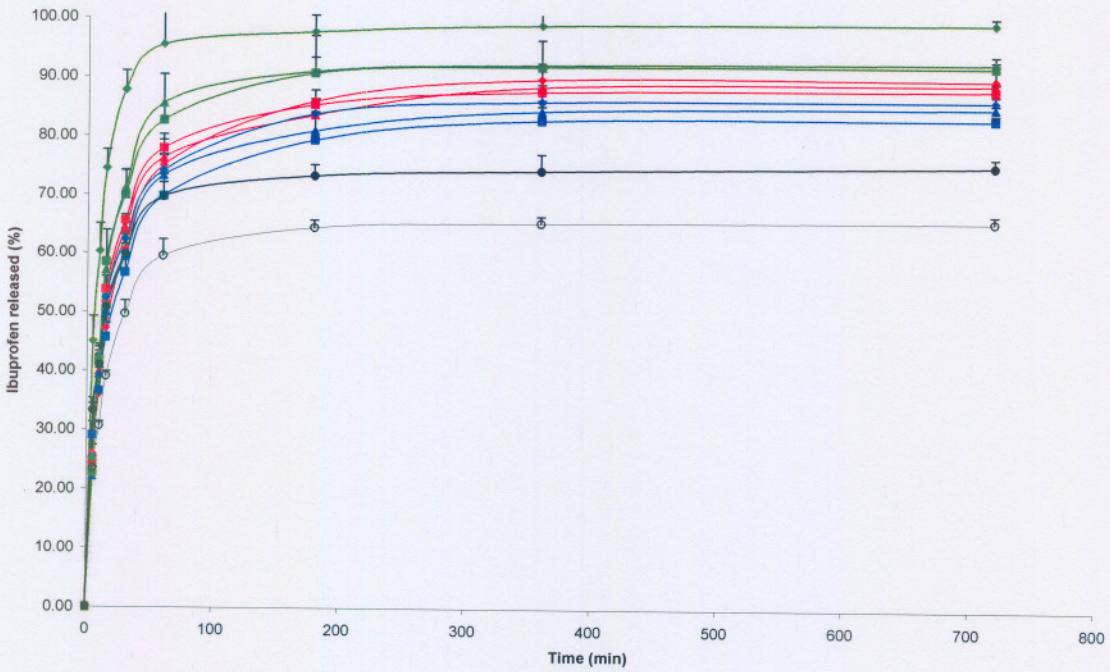


FIGURE 4.17 Dissolution profiles of ibuprofen from different TMC-H formulations. Legend: CHIT (○), CHIT-H (●), H-ADS-0.1 (■), H-ADS-0.2 (▲), H-ADS-0.5 (♦), H-EXP-0.1 (■), H-EXP-0.2 (▲), H-EXP-0.5 (♦), H-AA-0.1 (■), H-AA-0.2 (▲) and H-AA-0.5 (♦). Data is expressed as the mean ± SD (n=6).

TABLE 4.4 Difference factors (f_1) used to compare the change in dissolution profiles of different chitosan microbeads. Results for ibuprofen and TMC profiles are shown in the bottom left and top right part of the table respectively. Legend: red = no significant difference between profiles according to f_1 calculation, yellow = f_1 and f_2 values corresponding and blue = formulation with statistical significant different dissolution profiles from all other formulations tested.

	CHIT	CHIT-H	H-EXP-0.1	H-EXP-0.2	H-EXP-0.5	H-ADS-0.1	H-ADS-0.2	H-ADS-0.5	H-AA-0.1	H-AA-0.2	H-AA-0.5	CHIT-M	M-EXP-0.1	M-EXP-0.2	M-EXP-0.5	M-ADS-0.1	M-ADS-0.2	M-ADS-0.5	M-AA-0.1	M-AA-0.2	M-AA-0.5
CHIT		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CHIT-H	19.92		33.48	34.74	28.72	19.76	16.74	10.07	113.61	117.55	159.98	-	-	-	-	-	-	-	-	-	-
H-EXP-0.1	31.84	13.58		3.36	9.98	11.57	13.20	18.55	65.31	67.30	94.78	-	-	-	-	-	-	-	-	-	-
H-EXP-0.2	30.15	12.57	1.98		7.11	11.45	14.01	19.31	62.98	64.95	92.95	-	-	-	-	-	-	-	-	-	-
H-EXP-0.5	28.14	13.49	4.99	3.73		7.31	9.99	15.54	65.94	69.00	101.97	-	-	-	-	-	-	-	-	-	-
H-ADS-0.1	21.35	8.21	9.67	8.48	6.77		3.40	9.41	79.03	82.33	117.89	-	-	-	-	-	-	-	-	-	-
H-ADS-0.2	24.98	9.38	5.73	4.51	5.50	5.28		6.21	84.36	87.76	124.38	-	-	-	-	-	-	-	-	-	-
H-ADS-0.5	28.82	10.69	2.87	2.59	4.79	7.66	3.66		96.48	100.11	139.14	-	-	-	-	-	-	-	-	-	-
H-AA-0.1	38.52	19.57	5.65	6.84	9.01	16.43	11.13	8.72		2.65	21.71	-	-	-	-	-	-	-	-	-	-
H-AA-0.2	40.08	20.11	6.25	7.63	9.52	16.97	12.72	8.78	1.92		19.50	-	-	-	-	-	-	-	-	-	-
H-AA-0.5	65.50	38.01	25.53	27.16	29.15	36.38	33.16	28.47	19.83	18.14		-	-	-	-	-	-	-	-	-	-
CHIT-M	4.42	15.41	23.06	22.06	20.84	16.41	13.38	21.26	26.55	27.59	38.71			-	-	-	-	-	-	-	-
M-EXP-0.1	10.98	10.05	17.11	16.03	14.72	9.95	12.07	15.17	20.87	21.99	33.97	8.67		-	-	-	-	-	-	-	-
M-EXP-0.2	10.06	10.94	17.97	16.90	15.60	10.87	12.97	16.04	21.69	22.79	34.65	7.76	1.06		-	-	-	-	-	-	-
M-EXP-0.5	13.66	7.34	14.81	13.69	11.84	6.33	10.61	12.47	19.34	19.53	31.32	12.05	4.01	5.09		-	-	-	-	-	-
M-ADS-0.1	3.90	13.65	21.63	20.59	19.19	14.67	17.84	19.62	25.85	26.08	37.43	2.59	7.47	6.87	8.90		-	-	-	-	-
M-ADS-0.2	4.91	13.14	20.99	19.97	18.71	14.17	16.96	19.15	25.06	25.64	37.06	2.68	6.47	5.69	8.36	2.14		-	-	-	-
M-ADS-0.5	7.68	10.20	18.40	17.32	19.96	11.26	14.41	16.41	22.77	23.13	34.93	6.15	3.59	3.23	5.26	4.11	3.38		-	-	-
M-AA-0.1	23.66	5.15	8.27	7.07	9.10	5.93	5.75	5.99	13.10	13.38	25.28	21.91	13.16	14.34	8.80	19.42	18.72	14.84		-	-
M-AA-0.2	30.13	8.52	7.83	8.81	12.24	10.15	9.62	7.91	11.14	11.03	21.37	28.29	19.08	20.32	14.49	25.67	24.93	20.85	5.23		-
M-AA-0.5	32.44	10.44	6.08	7.47	10.88	9.73	8.58	6.56	8.90	8.83	19.97	30.56	21.19	22.46	16.52	27.90	27.15	22.99	7.10	2.37	

TABLE 4.5 Similarity factors (f_2) used to compare the change in dissolution profiles of different chitosan microbeads. Results for ibuprofen and TMC profiles are shown in the bottom left and top right part of the table respectively. Legend: red = no significant difference between profiles according to f_2 calculation, yellow = f_1 and f_2 values corresponding and blue = formulation with statistical significant different dissolution profiles from all other formulations tested.

	CHIT	CHIT-H	H-EXP-0.1	H-EXP-0.2	H-EXP-0.5	H-ADS-0.1	H-ADS-0.2	H-ADS-0.5	H-AA-0.1	H-AA-0.2	H-AA-0.5	CHIT-M	M-EXP-0.1	M-EXP-0.2	M-EXP-0.5	M-ADS-0.1	M-ADS-0.2	M-ADS-0.5	M-AA-0.1	M-AA-0.2	M-AA-0.5
CHIT		-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
CHIT-H	51.28		58.95	58.07	60.35	66.35	70.55	81.96	30.84	30.08	24.79	-	-	-	-	-	-	-	-	-	-
H-EXP-0.1	39.47	53.03		92.87	74.57	68.33	67.50	63.85	36.37	35.40	29.37	-	-	-	-	-	-	-	-	-	-
H-EXP-0.2	40.32	53.64	88.72		78.57	69.47	67.96	63.09	37.04	36.03	29.72	-	-	-	-	-	-	-	-	-	-
H-EXP-0.5	40.81	52.17	70.95	77.47		82.61	77.23	67.17	37.05	36.05	29.21	-	-	-	-	-	-	-	-	-	-
H-ADS-0.1	47.83	63.83	60.18	63.33	66.34		92.60	75.81	35.26	34.34	27.82	-	-	-	-	-	-	-	-	-	-
H-ADS-0.2	44.47	58.71	70.72	74.97	71.57	72.53		82.80	34.40	33.51	27.26	-	-	-	-	-	-	-	-	-	-
H-ADS-0.5	41.78	57.16	82.31	83.92	72.29	66.72	80.09		32.65	31.82	26.11	-	-	-	-	-	-	-	-	-	-
H-AA-0.1	35.18	45.70	70.36	66.32	60.13	50.41	56.79	61.87		90.84	51.15	-	-	-	-	-	-	-	-	-	-
H-AA-0.2	34.47	45.00	67.20	63.52	58.40	49.27	54.62	59.56	87.03		52.40	-	-	-	-	-	-	-	-	-	-
H-AA-0.5	25.39	33.03	39.38	38.17	36.31	33.46	35.11	37.59	42.79	44.35		-	-	-	-	-	-	-	-	-	-
CHIT-M	78.52	51.77	41.07	42.17	43.14	50.73	46.82	43.61	36.52	35.70	25.83		-	-	-	-	-	-	-	-	-
M-EXP-0.1	60.51	58.01	47.96	49.59	50.78	61.25	56.51	51.42	41.98	40.85	28.65	67.52		-	-	-	-	-	-	-	-
M-EXP-0.2	61.36	56.41	46.92	48.52	49.82	59.59	54.98	50.19	41.19	40.11	28.20	69.24	95.76		-	-	-	-	-	-	-
M-EXP-0.5	57.25	66.20	50.72	52.48	53.66	69.29	59.79	55.01	43.80	42.79	30.27	61.54	76.80	73.78		-	-	-	-	-	-
M-ADS-0.1	79.52	54.66	41.89	42.96	43.72	52.11	47.72	44.63	37.06	36.28	26.49	85.62	67.94	69.13	64.32		-	-	-	-	-
M-ADS-0.2	73.78	54.60	42.70	43.88	44.94	53.62	48.92	45.54	37.76	36.92	26.70	88.40	72.08	73.74	66.59	90.43		-	-	-	-
M-ADS-0.5	66.64	58.58	45.51	46.84	48.02	58.45	52.66	48.74	39.95	39.06	27.97	75.13	80.34	80.54	75.61	78.44	84.74		-	-	-
M-AA-0.1	47.17	74.35	61.86	62.57	59.57	70.31	68.39	67.30	51.76	50.72	35.15	48.47	56.50	54.83	63.05	50.23	50.85	54.83		-	-
M-AA-0.2	42.19	64.10	61.98	60.45	55.40	58.58	59.99	63.99	53.78	53.48	38.69	42.78	48.35	47.19	52.88	44.38	44.60	47.42	71.97		-
M-AA-0.5	40.49	59.15	67.18	64.51	58.06	58.08	61.09	67.33	58.26	58.05	40.28	41.31	47.02	45.94	51.05	42.65	43.03	45.76	68.18	85.19	

TABLE 4.6 Mean dissolution times (MDT in minutes) of ibuprofen and TMC release profiles from different chitosan microbead formulations.

Formulation	Mean Dissolution Time (min)	
	Ibuprofen	TMC
CHIT	11.60 ± 0.08	---
CHIT-H	10.74 ± 0.24	23.56 ± 0.76
H-EXP-0.1	12.81 ± 1.11	25.16 ± 1.50
H-EXP-0.2	15.42 ± 0.15	29.88 ± 1.73
H-EXP-0.5	16.04 ± 0.12	31.45 ± 1.06
H-ADS-0.1	15.98 ± 0.95	25.68 ± 2.12
H-ADS-0.2	15.21 ± 0.20	27.74 ± 1.53
H-ADS-0.5	13.81 ± 0.83	20.20 ± 1.84
H-AA-0.1	11.18 ± 0.33	23.58 ± 1.12
H-AA-0.2	10.44 ± 0.13	22.18 ± 2.27
H-AA-0.5	7.15 ± 0.56	15.45 ± 1.55
CHIT-M	15.01 ± 0.10	---
M-EXP-0.1	17.24 ± 0.19	---
M-EXP-0.2	17.75 ± 0.13	---
M-EXP-0.5	17.80 ± 0.18	---
M-ADS-0.1	16.30 ± 0.32	---
M-ADS-0.2	15.71 ± 0.49	---
M-ADS-0.5	14.79 ± 0.24	---
M-AA-0.1	10.21 ± 0.16	---
M-AA-0.2	7.46 ± 0.20	---
M-AA-0.5	7.96 ± 0.11	---

4.2.2.2.5 Conclusion

The results obtained from the inclusion of different additives to the chitosan microbead formulations lead to the following conclusions:

- The inclusion of either TMC-M or TMC-H and different concentrations of either Ac-di-sol®, Explotab® or ascorbic acid to the chitosan microbead formulation lead to no significant morphological change in the appearance of the beads.
- The plain chitosan control loaded the highest percentage of ibuprofen while no significant differences were obtained for the ibuprofen content (% DC) between the formulations containing TMC-M or TMC-H.
- The loading and resultant % DC of TMC-M was overall less than that of TMC-H while little differences were found within the formulations containing either TMC-M or TMC-H.
- No significant differences in the percentage degree of swelling were obtained between the formulations containing either TMC-M or TMC-H, although the beads containing TMC-M swelled overall less than those with TMC-H included. All the bead variants swelled significantly more than the chitosan control.
- Dissolution studies indicated that very little TMC-M were released from all formulations while TMC-H were released significantly more from the beads containing ascorbic acid.
- The release of ibuprofen was less from the formulations containing TMC-M and the highest from the formulation containing TMC-H and 0.5 % (w/v) ascorbic acid.

It is therefore evident that the TMC-M polymer (medium molecular weight and high degree of quaternisation) revealed poorer inclusion and release characteristics than TMC-H (high molecular weight and medium degree of quaternisation). It is further noted that the inclusion of different additives in different concentrations resulted in no significant change in the release of TMC

and ibuprofen, except for the formulations containing ascorbic acid. The formulation containing TMC-H and 0.5 % ascorbic acid resulted in the highest release of both TMC-H and ibuprofen from the chitosan microbeads.

4.3 CONCLUSION

Chitosan microbeads with either TMC-M or TMC-H were successfully prepared by ionotropic gelation. The most suitable concentration of TMC in the chitosan microbeads were determined by scanning electron microscopy, loading capacity and swelling behaviour. It was found that the concentration of TMC used in the preparation of the beads influenced the morphology and integrity of the beads. A proportional relationship was found for both TMC polymers between the concentration TMC added, the percentage TMC loading capacity (% DLC) and the percentage degree of swelling of the microbeads at pH 6.8. The degree of swelling and % DLC increased as the concentration TMC increased. The results obtained illustrated that 2 % (w/v) TMC added to the chitosan solution showed much potential to aid with the inclusion-ion of a model drug compound and other additives within the microbeads.

Ibuprofen and different concentrations of the pharmaceutical excipients Ac-di-sol®, Explotab® and ascorbic acid were loaded within the microbeads. Scanning electron micrographs showed no significant differences in the morphology of different formulations. The loading capacity of the TMC-M polymer was significantly lower than that of TMC-H while the loading of ibuprofen remained at approximately 15 to 20 % irrespective of the additives or TMC polymer used. An increased TMC loading capacity resulted in increased swelling behaviour of the chitosan microbeads at pH 6.8. During dissolution studies on the different microbead formulations the release of TMC-M was below the limit of detection due to the poor loading capacity. The release of TMC-H was significantly higher from the microbeads containing the 0.5 % (w/v) ascorbic acid, releasing almost 70 % of the TMC. The formulations containing TMC-H released more ibuprofen

than those containing TMC-M. Again the TMC-H formulations containing ascorbic acid released the highest quantity of ibuprofen with the 0.5 % (w/v) ascorbic acid formulation releasing almost 100 % of the ibuprofen.

It is therefore evident that the TMC-H polymer showed superior inclusion and release properties over TMC-M from chitosan microbeads while the inclusion of 0.5 % (w/v) ascorbic acid resulted in the highest TMC and ibuprofen release. The chitosan microbead formulation containing 2 % (w/v) TMC-H and 0.5 % (w/v) ascorbic acid will therefore be used for the *in vitro* absorption studies in chapter 5.

CHAPTER 5

IN VITRO ABSORPTION STUDIES WITH CHITOSAN MICROBEADS

Chitosan is a biodegradable natural polymer with great potential for pharmaceutical applications due to its biocompatibility, high charge density, non-toxicity and mucoadhesion. Gel formation can be obtained by interactions of chitosans with low molecular counterions such as polyphosphates which result in bead formation. This gelling property of chitosan allows a wide range of applications such as coating of pharmaceuticals, gel entrapment of biochemicals, plant embryos, whole cells, micro-organisms and algae.

In order to increase the absorption of poorly permeable drugs, excipients such as absorption enhancers have been evaluated. *N*-trimethyl chitosan chloride (TMC) is a partially quaternised derivative of chitosan with superior solubility compared with other chitosan salts. These polymers enhance the absorption of hydrophilic molecules through intestinal epithelia by triggering the reversible opening of tight junctions and only allow for paracellular transport (Thanou *et al.*, 2000a:23; Kotzé *et al.*, 1997a:251). For TMC to be active as an absorption enhancer, it should be in solution. Therefore, the amount of the polymer released from the dosage form should be determined parallel to the amount of drug released as evidenced in chapter 4. In chapter 4 the loading and release of TMC from microbeads was shown. In order to test the hypothesis it was necessary to perform *in vitro* permeation studies using rat jejunum to determine the effectiveness of TMC as a potential absorption enhancer of hydrophilic compounds.

In this chapter the validation of a high pressure liquid chromatographic (HPLC) analysis for ibuprofen will be described. A HPLC method of analysis was chosen instead of the UV spectrophotometric analysis as used and described in chapter 4, since more sensitive methods of analysis are needed when working with biological membranes in *in vitro* permeation studies.

The absorption enhancing properties of TMC from a solid dosage form was also studied *in vitro*. The chitosan microbead formulation containing 2 % (w/v) TMC-H and 0.5 % (w/v) ascorbic acid was selected for these *in vitro* studies since this formulation showed the highest release of TMC and ibuprofen (see chapter 4). Chitosan containing only TMC-H (no ascorbic acid) and a plain chitosan microbead formulation was used as control formulations during the *in vitro* absorption studies.

5.1 VALIDATION OF A HIGH PRESSURE LIQUID CHROMATOGRAPHIC (HPLC) ANALYSIS FOR IBUPROFEN

The objective was to develop an analytical method that was easy to use and sensitive enough for the quantitative determination of ibuprofen in phosphate buffered solution (PBS), following permeation through rat jejunum.

5.1.1 Reagents and raw materials

Ibuprofen (annexure 4) was purchased from Sandoz (South Africa). HPLC analytical grade acetonitrile (CH_3OH) (Merck Co., Midrand, South Africa) and ortho-phosphoric acid (H_3PO_4) 85 % AR at pH 1.5 (ACE, South Africa) were used. All other reagents were reagent grade (Merck Co., Midrand, South Africa) and were used as received. Phosphate buffer solution (PBS) at pH 6.8 was prepared according to the USP (2004:2724) method. Double distilled deionised water was prepared by a Milli-Q Academic water purification system (Millipore, Milford, USA). Annexure 5 shows the certificate of analysis for flufenamic acid purchased from Sigma (Jetpark, South Africa).

5.1.2 Analysis in phosphate buffer solution (PBS)

5.1.2.1 High pressure liquid chromatography (HPLC) system

The HPLC system that was used for analysis of ibuprofen in PBS consisted of a SpectraSYSTEM P1000 pump (Thermo Separation Products (TSP), California, USA), SpectraSYSTEM AS 3000 Autosampler (TSP, California, USA), SpectraSYSTEM UV variable wavelength UV-detector (TSP, California, USA) and a SpectraSYSTEM SN 4000 attached to a PC 1000 software integrator (TSP, California, USA).

5.1.2.2 Chromatographic conditions

The analysis method described in the BP (2004:1010) was adapted for this HPLC system and to include flufenamic acid as internal standard.

HPLC column:	Phenomenex® Luna C18 (2), 5 µm, 250 x 4.60 mm stainless steel column (Phenomenex, USA).
Pre-column:	LiChroCART® 4-4, LiCrospher® 100 RP-18, 5 µm, guard columns (Merck, Darmstadt, Germany).
Mobile phase:	The mobile phase consisted of acetonitrile-water (550:450 v/v) to which were added 9.86 g ortho-phosphoric acid per liter mobile phase. The apparent pH was adjusted to 4.5 with 5 N sodium hydroxide (NaOH). The mobile phase was degassed by filtration through a Micron-PES, polysulfone, 0.45 µm filter (Osmonics Inc., USA).
Flow rate:	1.5 ml/min.
Injection volume:	5 µl.
Detection:	UV at 220 nm.

Internal standard: Flufenamic acid.

Retention times: ± 9.7 minutes for ibuprofen and ± 12.0 minutes for flufenamic acid.

Temperature: ± 25 °C.

5.1.2.3 Preparation of standard solutions

50 mg of ibuprofen was weighed accurately, transferred into a 100 ml volumetric flask and dissolved in prepared mobile phase. The flask was filled to volume with mobile phase, to produce a 500 µg/ml stock solution. Standard solutions were prepared by transferring the required volume from this stock solution analytically to a volumetric flask and adding prepared PBS to volume as shown in table 5.1.

A solution of flufenamic acid was prepared by weighing 50 mg of the raw material accurately and transferring it to a 100 ml volumetric flask. The flufenamic acid was dissolved in PBS and the flask filled to volume with PBS. This produced a stock solution with a concentration of 500 µg/ml. This flufenamic acid stock solution (500 µg/ml) was then used to produce the various ibuprofen standard solutions as shown in table 5.1, with flufenamic acid (20 µg/ml) as internal standard.

5.1.2.4 Sample preparation

A solution of flufenamic acid was prepared by weighing 22 mg of the raw material accurately and transferring it to a 100 ml volumetric flask. The flufenamic acid was dissolved in mobile phase and the flask filled to volume with additional mobile phase. This produced a stock solution with a concentration of 220 µg/ml. From this flufenamic acid stock solution (220 µg/ml) 20 µl was then transferred to 200 µl of each of the ibuprofen sample solutions. This produced flufenamic acid

as internal standard to each of the sample solutions with a concentration of 20 µg/ml.

TABLE 5.1 Dilutions made from the stock solution (500 µg/ml) and the concentrations (µg/ml) used for the linear regression curve.

Concentration (µg/ml) of standard solutions	Volume (ml) of ibuprofen stock solution transferred	Volume (ml) of flufenamic acid stock solution transferred	Volume (ml) of volumetric flask
10.0	1.0	2.0	50.0
20.0	2.0	2.0	50.0
30.0	3.0	2.0	50.0
40.0	4.0	2.0	50.0
50.0	5.0	2.0	50.0

5.1.2.5 Column maintenance

After performing an analysis, at least 30 ml of 50/50 methanol/water was passed through the chromatograph (flow rate of 1 ml/min). The system was then rinsed with at least 30 ml ammonium sulphate solution (0.07 M) at a flow rate of 1 ml/min before storing the column.

5.1.2.6 Experimental procedures

5.1.2.6.1 Linearity

Preparation of standards

Linear regression of ibuprofen analysis was performed using the peak area ratio (standard/internal standard) versus concentration of the standard. Five standard solutions were prepared as described in § 5.1.2.3 by diluting a stock solution

(500 µg/ml) to obtain standard concentrations ranging from 10 to 50 µg/ml. To determine intra-day variation for linearity, three separate sets of standard solutions were prepared and analysed on the same day in order to compare their linear regression. Inter-day variation for linearity was determined by preparing and analysing three separate sets of standard solutions on three different days, to compare their linear regression. During the experimental analysis, standard curves were constructed daily. Figure 5.1 shows an example of a linear regression curve obtained for ibuprofen with flufenamic acid as internal standard.

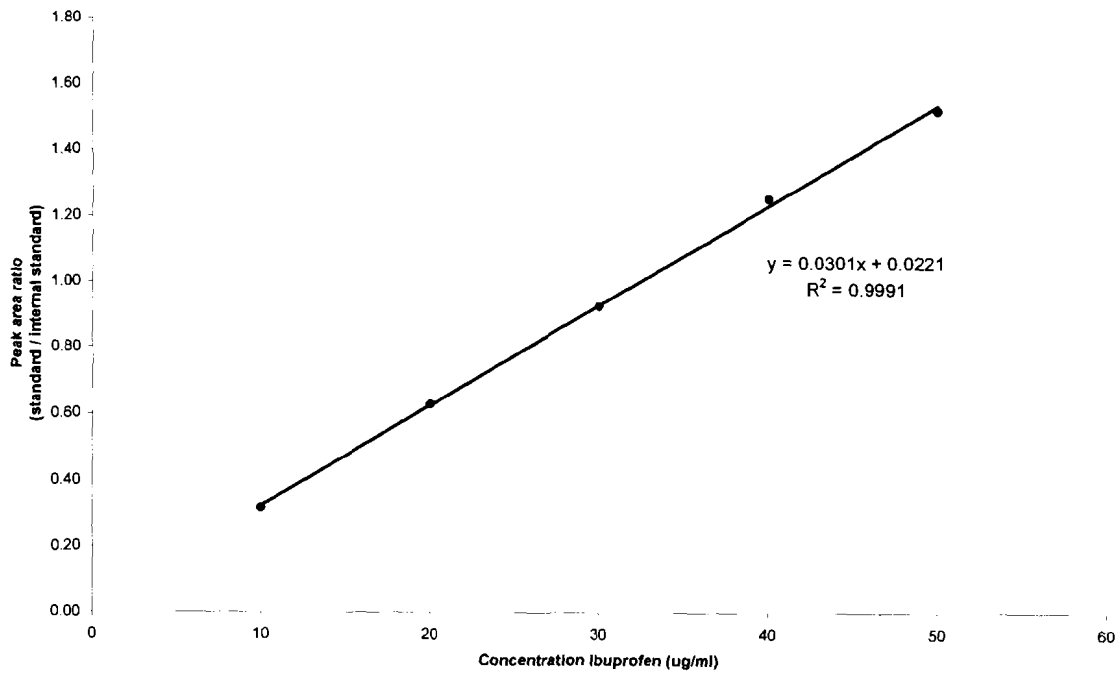


FIGURE 5.1 Linear regression curve for ibuprofen.

Intra-day linearity

TABLE 5.2 The peak area ratio (standard/internal standard) obtained from 3 different linear regression curves on the same day.

Concentration ibuprofen (µg/ml)	Peak area ratio (standard/internal standard)			Mean	SD
	Calibration curve 1	Calibration curve 2	Calibration curve 3		
10.0	0.3057	0.3168	0.3143	0.3123	0.0058
20.0	0.6249	0.6281	0.6281	0.6270	0.0018
30.0	0.9494	0.9233	0.9479	0.9402	0.0147
40.0	1.2218	1.2335	1.2456	1.2336	0.0119
50.0	1.5167	1.5128	1.5110	1.5135	0.0029

TABLE 5.3 The mean R^2, y-intercept and slope of the regression curves to determine intra-day variability.

R-squared (R^2)	0.9991
Y-intercept	0.0226
Slope	0.0301

Inter-day linearity

TABLE 5.4 The peak area ratio (standard/internal standard) obtained from 3 different linear regression curves on 3 consecutive day.

Concentration ibuprofen (µg/ml)	Peak area ratio (standard/internal standard)			Mean	SD
	Calibration curve 1	Calibration curve 2	Calibration curve 3		
10.0	0.3101	0.3142	0.3173	0.3139	0.0036
20.0	0.6229	0.6241	0.6262	0.6244	0.0017
30.0	0.9388	0.9442	0.9466	0.9432	0.0040
40.0	1.2323	1.2291	1.2394	1.2336	0.0053
50.0	1.5118	1.5173	1.5146	1.5146	0.0028

TABLE 5.5 The mean R^2, y-intercept and slope of the regression curves to determine inter-day variability.

R-squared (R^2)	0.9993
Y-intercept	0.0227
Slope	0.0301

Conclusion

The method was found to be linear over the concentration range of 10 to 50 µg/ml.

5.1.2.6.2 Accuracy

Accuracy was determined by analysing 3 samples with known amounts of ibuprofen at a low (10 µg/ml), intermediate (30 µg/ml) and high (50 µg/ml) concentration, giving a total of 9 samples.

TABLE 5.6 Percentage (%) ibuprofen recovered by analysing 3 samples (low – 10 µg/ml, intermediate - 30 µg/ml and high - 50 µg/ml).

	Low concentration (µg/ml)			Intermediate concentration (µg/ml)			High concentration (µg/ml)		
Conc. spiked	10.0	10.0	10.0	30.0	30.0	30.0	50.0	50.0	50.0
Conc. found	9.99	9.77	9.69	30.06	29.79	29.71	50.27	50.17	49.91
% Recover	99.92	97.68	96.88	100.21	99.29	99.04	100.53	100.34	99.82

TABLE 5.7 The mean, standard deviation (SD) and percentage relative standard deviation (% RSD) for the percentage (%) ibuprofen recovered by analysing 3 samples (low - 10 µg/ml, intermediate - 30 µg/ml and high - 50 µg/ml).

	Low concentration (µg/ml)	Intermediate concentration (µg/ml)	High concentration (µg/ml)
Mean	98.16 %	99.51 %	100.23 %
SD	1.58	0.62	0.37
% RSD	1.41	0.33	0.24

Conclusion

A mean recovery between 97.0 and 103.0 % was achieved over the concentration range tested and are therefore acceptable.

5.1.2.6.3 Precision

Intra-day precision

Precision (or repeatability) was determined by preparing 3 sets of 3 samples with known amounts of ibuprofen at a low (10 µg/ml), intermediate (30 µg/ml) and high (50 µg/ml) concentration.

TABLE 5.8 Percentage (%) ibuprofen recovered by analysing 3 sets of 3 samples (low – 10 µg/ml, intermediate - 30 µg/ml and high - 50 µg/ml) on the same day.

	Low concentration (µg/ml)			Intermediate concentration (µg/ml)			High concentration (µg/ml)		
Conc. spiked	10.0	10.0	10.0	30.0	30.0	30.0	50.0	50.0	50.0
Conc. found	9.99	10.07	9.81	29.53	30.21	30.43	50.08	50.22	49.66
% Recover	99.95	100.69	98.12	98.43	100.69	101.44	100.15	100.44	99.31

TABLE 5.9 The mean, standard deviation (SD) and percentage relative standard deviation (% RSD) for the percentage (%) ibuprofen recovered by analysing 3 sets of 3 samples (low - 10 µg/ml, intermediate - 30 µg/ml and high - 50 µg/ml) on the same day.

	Low concentration (µg/ml)	Intermediate concentration (µg/ml)	High concentration (µg/ml)
Mean	99.59 %	100.19 %	99.97 %
SD	1.32	1.57	0.59
% RSD	1.01	1.04	0.57

Inter-day precision

The same sample concentrations were analysed in triplicate on three different occasions to determine the between-day variability of the method.

TABLE 5.10 Percentage (%) ibuprofen recovered by analysing 3 sets of 3 samples (low – 10 µg/ml, intermediate - 30 µg/ml and high - 50 µg/ml) on 3 consecutive days.

	Low concentration (µg/ml)			Intermediate concentration (µg/ml)			High concentration (µg/ml)		
Conc. spiked	10.0	10.0	10.0	30.0	30.0	30.0	50.0	50.0	50.0
Conc. found	9.82	9.94	9.92	29.99	29.69	29.55	50.21	49.46	49.92
% Recover	98.22	99.36	99.21	99.96	98.98	98.50	100.42	98.92	99.84

TABLE 5.11 The mean, standard deviation (SD) and percentage relative standard deviation (% RSD) for the percentage (%) ibuprofen recovered by analysing 3 sets of 3 samples (low - 10 µg/ml, intermediate - 30 µg/ml and high - 50 µg/ml) on 3 consecutive days.

	Low concentration (µg/ml)	Intermediate concentration (µg/ml)	High concentration (µg/ml)
Mean	98.93 %	99.15 %	99.73 %
SD	0.62	0.74	0.76
% RSD	0.53	0.67	0.69

Conclusion

The within-day and inter-day precision was found to be acceptable.

5.1.2.6.4 Selectivity

Selectivity is the ability of the analytical method to detect and analyse a component in the presence of other components such as decomposition products as well as biological materials. Figure 5.2 shows a chromatograph obtained for ibuprofen (30 µg/ml) with flufenamic acid (20 µg/ml) as internal standard. Figure 5.3 shows a chromatograph obtained for a blank phosphate buffer solution without any of the two drugs spiked. This method seems to be selective since there are no endogenous interfering peaks at the retention time of ibuprofen (8 to 10 minutes) and flufenamic acid (11 to 13 minutes).

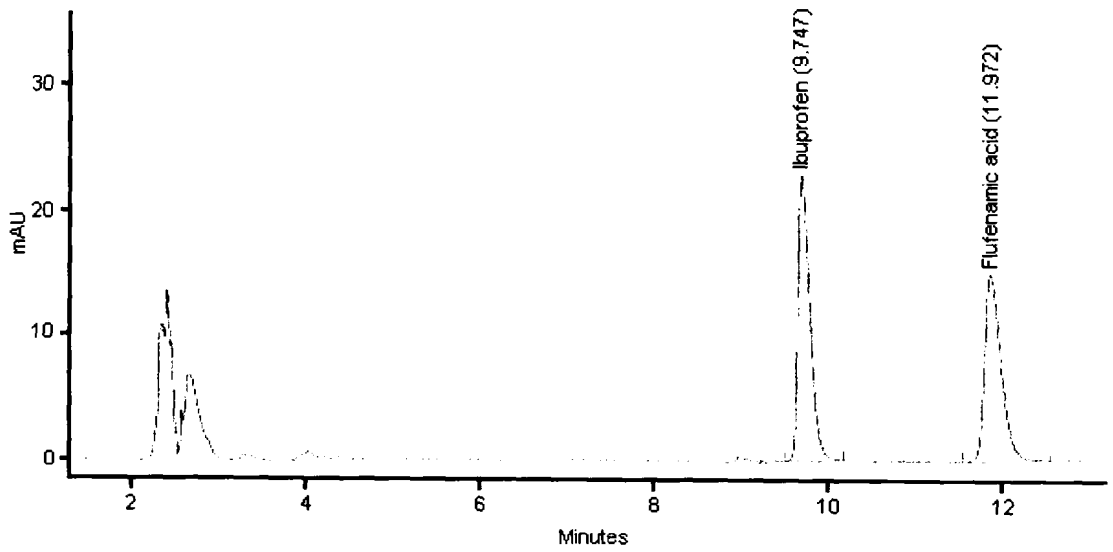


FIGURE 5.2 HPLC-chromatogram obtained for ibuprofen (30 µg/ml) with flufenamic acid (30 µg/ml) as internal standard.

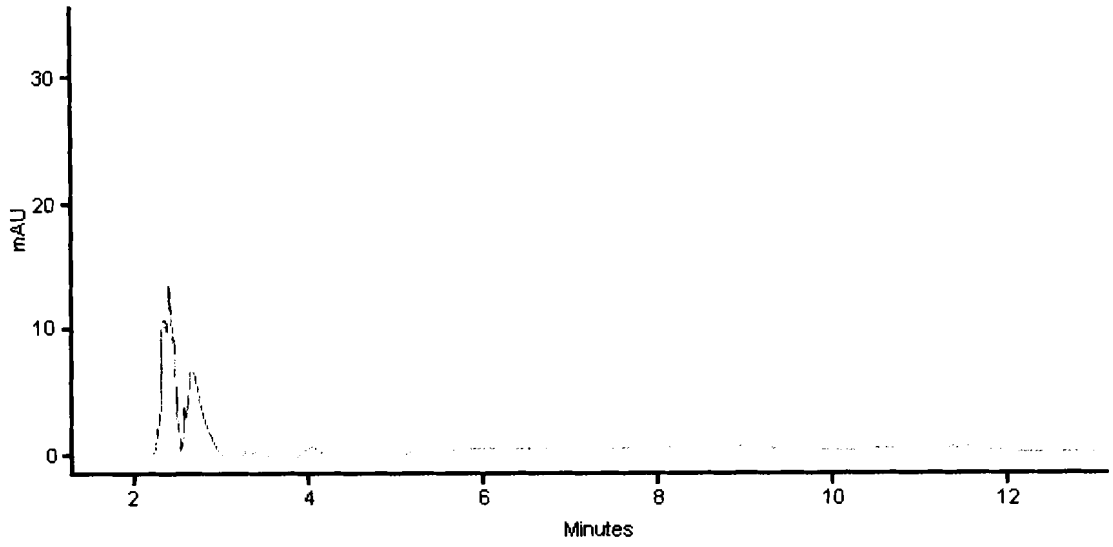


FIGURE 5.3 HPLC-chromatogram obtained for a blank phosphate buffer solution (PBS) without any drugs spiked.

5.1.2.6.5 Sensitivity

The sensitivity of an analytical method can be measured by determining the limit of quantification and limit of detection. The limit of quantification is defined as the lowest concentration of an analyte in a sample that can be determined with acceptable precision and accuracy (% RSD < 15 %). The limit of detection, on the other hand, is defined as the lowest concentration of analyte in a sample that can be detected, but not necessarily quantified. Six different samples of a specific ibuprofen concentration (5 µg/ml) were analysed on the same day to determine the limit of quantification.

TABLE 5.12 The peak area ratio (standard/internal standard) of six different samples of a specific ibuprofen concentration (5 µg/ml) to determine the limit of quantification.

Concentration ibuprofen (µg/ml)	Ibuprofen peak area (AUC)	Concentration flufenamic acid (µg/ml)	Flufenamic acid peak area (AUC)	Peak area ratio (standard / internal standard)
5.0	34914	20.0	232233	0.1503
5.0	36781	20.0	235804	0.1560
5.0	36424	20.0	235107	0.1549
5.0	34081	20.0	231764	0.1471
5.0	35233	20.0	233197	0.1511
5.0	36473	20.0	235146	0.1551

TABLE 5.13 The mean, standard deviation (SD) and percentage relative standard deviation (% RSD) of six different samples of a specific ibuprofen concentration (5 µg/ml) to determine the limit of quantification.

Mean	0.1524
SD	0.0035
% RSD	1.56

Conclusion

Based on a 200 µl sample volume, the lowest quantifiable concentration for ibuprofen was 5 µg/ml (% RSD < 15 %) and the limit of detection for ibuprofen was 1 µg/ml.

5.1.2.6.6 Stability of sample solutions

A sample (30 µg/ml) was left on the autosampler tray and reanalysed every 2 hours over a twelve-hour period to determine the stability of the samples.

TABLE 5.14 Stability of ibuprofen over a period of 12 hours.

Time (hours)	Peak area (AUC)	Percentage recover (%)
0	218721	100.0
2	217190	99.3
4	216096	98.8
6	214347	98.0
8	214346	98.0
10	213690	97.7
12	212815	97.3

Conclusion

The samples proved to be stable in an amber vial when left in the autosampler for at least twelve hours. All samples and standards were analysed within twelve hours after they were put in the autosampler.

5.1.2.6.7 System repeatability

A sample (30 µg/ml) was injected six times in order to test the repeatability of the peak area as well as the retention time.

TABLE 5.15 Repeatability of the peak area (AUC) and retention time (minutes) for ibuprofen.

	Peak area (AUC)	Retention time (minutes)
	218721	9.745
	217081	9.747
	219901	9.745
	218128	9.748
	219455	9.745
	218907	9.752
Mean	218699	9.747
% RSD	1.42	5.36×10^{-4}

5.1.2.6.8 Peak symmetry

The tailing factor, T, a measure of peak symmetry, is unity for perfectly symmetrical peaks and its value increases as tailing becomes more pronounced. As peak symmetry increases, integration and hence precision becomes less reliable. The general requirement is that T does not exceed the value of 2.0. Figure 5.4 shows a diagram of the parameters used to calculate tailing and the degree of tail or head formation was calculated with equation 6 (USP, 2004:2281).

The tailing factors (T) for ibuprofen and flufenamic acid was determined to be:

Ibuprofen: T = 1.2164
Flufenamic acid: T = 1.3982

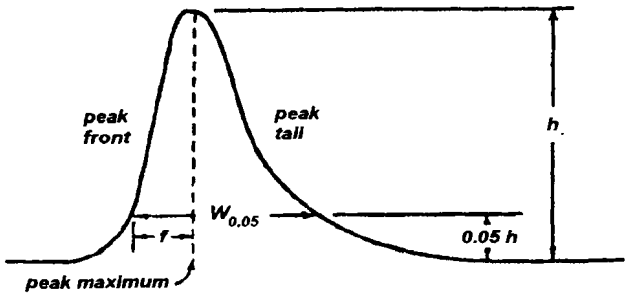


FIGURE 5.4 Diagram of parameters used to calculate tailing (USP, 2004:2281).

$$T = \frac{W_{0.05}}{2f} \tag{6}$$

where:

T = tailing factor,

h = height of peak,

$W_{0.05}$ = width of peak at 5 % height,

f = the leading edge of the peak, the distance being measured at a point 5 % of the peak height from the baseline.

As shown from the tailing factors determined, ibuprofen and flufenamic acid did not show appreciable tailing (< 2.0).

5.1.2.6.9 Resolution (R)

Resolution describes the degree or magnitude of the separation between two peaks. Resolution can be defined mathematically as the difference in elution volumes of two components, divided by their average band widths. This can be measured from a chromatogram as shown in figure 5.5. The values of t_1, t_2, W_1 and W_2 may be measured in units of volume, time or distance. Whichever is used, consistency is the rule, as all units must cancel to give a dimensionless

value of resolution. The resolution was calculated with equation 7 (USP, 2004:2280).

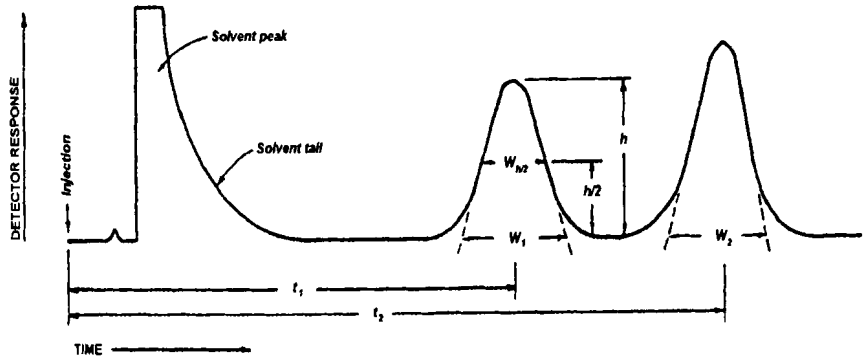


FIGURE 5.5 Diagram of parameters used to calculate resolution (USP, 2004:2280).

$$R = \frac{t_2 - t_1}{0.5(W_1 + W_2)} \quad (7)$$

where:

t_1 = retention time for ibuprofen,

t_2 = retention time for flufenamic acid,

W_1 = ibuprofen corresponding width at the base of the peak,

W_2 = flufenamic acid corresponding width at the base of the peak.

The resolution between the ibuprofen and flufenamic acid (internal standard) peaks was 6.37. R should be greater than 1.5 for peaks to be well resolved. According to the resolution determined, the peaks were well resolved.

5.2 *IN VITRO* PERMEATION STUDIES ACROSS RAT JEJUNUM

Prediction of the ability of a drug molecule to transverse an epithelial barrier is difficult to make from simple physico-chemical measurements, such as pK_a,

molecular size (or weight) and partition coefficient, which tend to oversimplify the driving forces for transport. Direct measurement of tissue permeability would be advantageous in ascertaining whether problems in drug absorption are the result of low solubility or poor tissue permeability (Grass and Sweetana, 1988:372). The effect of *N*-trimethyl chitosan chloride (TMC-H, M_W = 233 700 g/mole and degree of quaternisation = 48.5 %) on the permeability/transport of ibuprofen across rat intestine was investigated using a vertical diffusion chamber system as previously described by Swart (2002:26). The experimental setup, derived from the Ussing chamber, comprised of six Sweetana-Grass diffusion chambers, one heating block and a gas manifold (Corning Costar Corporation, Cambridge, USA) (figure 5.6).

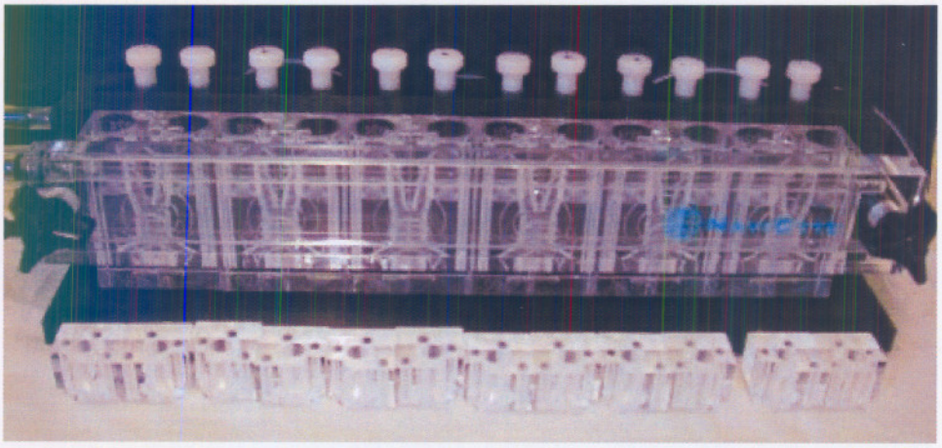


FIGURE 5.6 Sweetana-Grass diffusion chambers, heating block and gas manifold used for the *in vitro* permeation studies of ibuprofen across rat jejunum (Swart, 2002:26).

This experimental method was chosen above Caco-2 cell lines, even though Caco-2 cell lines are one of the most commonly used methods for transport studies. Some of the disadvantages of Caco-2 cell lines relate to the cost involved, time taken to culture the cells (24 to 27 days) and the sterile conditions under which the cells must be maintained (Cogburn *et al.*, 1991:210). Models using rat intestine are faster and cheaper to use and should potentially be more representative of the true absorption process.

5.2.1 Materials

The chitosan microbeads containing ibuprofen, TMC-H and ascorbic acid were used as synthesised in chapter 4. The Fijian-000201 chitosan (83 % deacetylated) (annexure 3) were purchased from Edelweis Pharmaceuticals (Johannesburg, South Africa). Ibuprofen raw material (annexure 4) was purchased from Sandoz (Isando, South Africa) and TMC-H (M_W = 233 700 g/mole and degree of quaternisation = 48.5 %) was synthesised as described in chapter 3. Ascorbic acid was purchased from Merck Co. (Midrand, South Africa), while Krebs-Ringer bicarbonate buffer were obtained from Sigma (Johannesburg, South Africa). Phosphate buffer solution (PBS) at pH 6.8 was prepared according to the USP (2004:2724) method. All other reagents were obtained from Merck Co (Midrand, South Africa) and were used as received. All water used was distilled and deionised.

5.2.2 Method of tissue preparation

Previous studies by Swart (2002:27) found it necessary to remove the serosal muscle layer, as it reduced and in some cases completely prevented the transport of drugs when using *in vitro* experimental models.

Unfasted adult male Spargue-Dawley rats (350 to 450 g) (obtained from the Animal Centre, North-West University, Ethical Committee approval number 04D14) (annexure 6) were sacrificed by halothane inhalation. After an abdominal incision was made a strip of intestinal tissue (jejunum, 20 to 30 cm) was excised starting approximately 5 cm from the stomach. The tissue was rinsed with ice cold Krebs-Ringer bibarbonate buffer (KR) (bubbled with a mixture of 95 % O_2/ 5 % CO_2) and then pulled onto a glass rod (figure 5.7). The back of a scalpel was used to gently scour the excised tissue along the mesenteric border. Removal of the serosal muscle layer was then obtained by gentle rubbing with the forefinger along the mesenteric border. Throughout the procedure, the tissue was immersed in ice cold KR which was kept in an ice bath. The excised strip

was then cut along the mesenteric border and transferred onto a strip of filter paper.

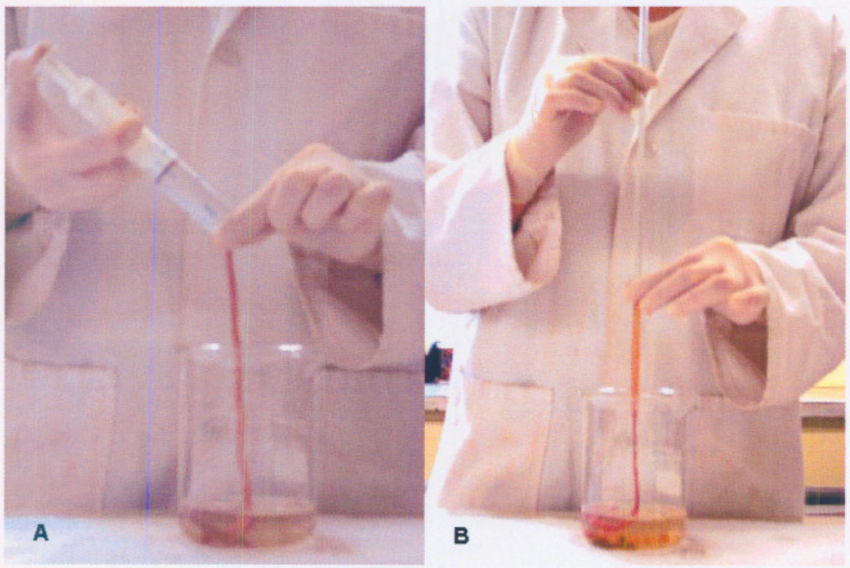


FIGURE 5.7 Rinsing the excised intestinal tissue (A) and pulling it onto a glass rod (B) (Swart, 2002:28).

Intestinal tissue with lengths of approximately 3 cm was then cut from the strip on ice and kept moist with ice cold KR (figure 5.8). Care was taken to avoid segments containing Payer's patches. Payer's patches are lymph like tissues which might cause greater variation in transport rates because of their altered morphology and the thickness of the epithelial layer.

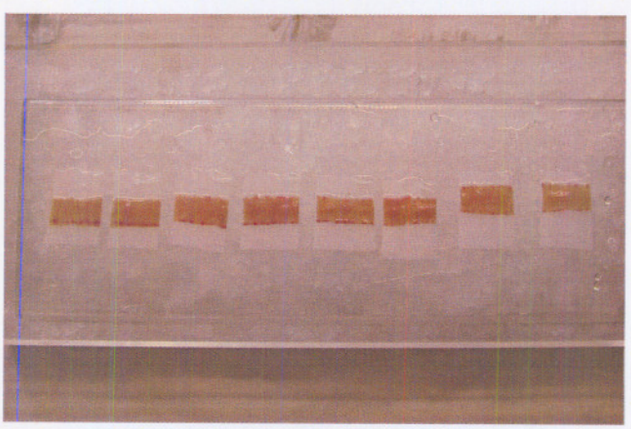


FIGURE 5.8 Segments of rat intestinal tissue (jejunum) used in Sweetana-Grass diffusion chambers (Swart, 2002:29).

The segments were carefully mounted onto the pins of each half-cell (preheated to 37 °C) immediately after tissue preparation (figure 5.9).

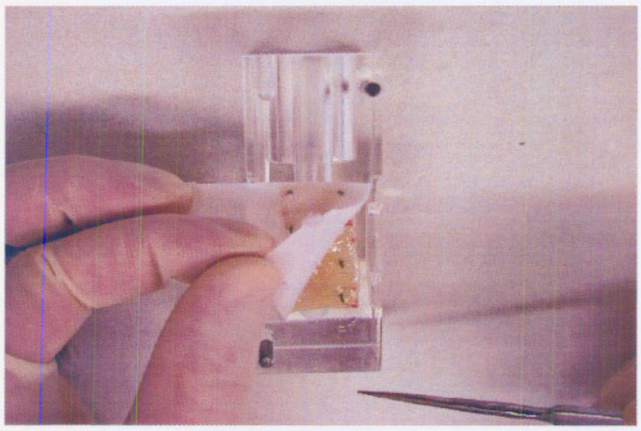


FIGURE 5.9 Mounting of the intestinal tissue on each half-cell (Swart, 2002:30).

The matching half-cells were then carefully clamped together without damaging the jejunal membrane (figure 5.10) and the assembled chamber was placed in the heating block (preheated to 37 °C).

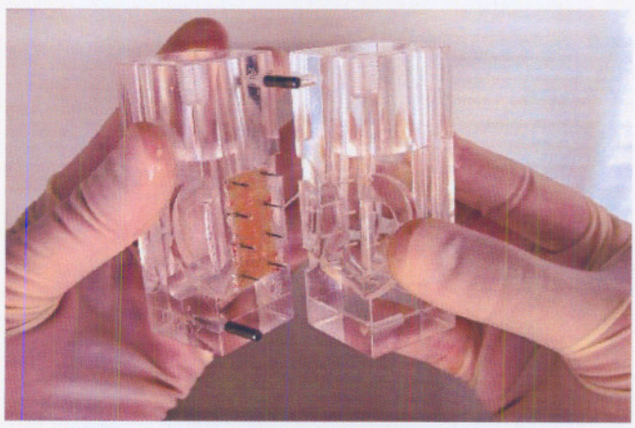


FIGURE 5.10 Assembling of the Sweetana-Grass diffusion chambers (Swart, 2002:30).

From the prepared phosphate buffer solution (PBS, pH 6.8, preheated to 37 °C) 5 ml was added to the donor and receiver compartment of each chamber while circulation of the buffer was maintained by a gas lift (95 % O_2/5 % CO_2, flow rate

15 to 20 ml/min.). Six chambers were assembled and placed in the heating block as shown in figure 5.6. The tissue was allowed to acclimatise for 15 minutes before any transport study was started. The preparation of the tissue and chambers took approximately 25 minutes. The cells were kept in the heating block at 37 °C for the duration of each experiment.

5.2.3 Experimental procedure

The microbead formulation which exhibited the highest potential for the release of both TMC-H and ibuprofen (discussed in chapter 4) were chosen to be investigated for its *in vitro* permeation enhancement capabilities. Chitosan microbeads containing 2 % (w/v) ibuprofen, 2 % (w/v) TMC-H and 0.5 % (w/v) ascorbic acid (H-AA-0.5) were compared with (1) a formulation containing only 2 % (w/v) ibuprofen and 2 % (w/v) TMC-H (CHIT-H) and (2) a plain ibuprofen loaded chitosan microbead (CHIT) as controls. Microbeads equivalent to 50 mg ibuprofen (determined from percentage drug content (% DC) as discussed in chapter 4) were added from each formulation to the donor compartment and another 2 ml PBS (pH 6.8) was added to both the receiver- (apical side) and donor cell (basolateral side). Table 5.16 shows the quantity of ibuprofen and TMC-H used, as determined from the individual % DC for each microbead formulation studied.

TABLE 5.16 Quantity (mg) of ibuprofen and TMC-H used for each microbead formulation studied.

Formulation	Weight (mg) of beads used	% DC of Ibuprofen	Mass (mg) of ibuprofen	% DC of TMC-H	Mass (mg) of TMC-H
CHIT	180.0	27.81	50.0	---	---
CHIT-H	244.0	20.48	50.0	17.47	42.6
H-AA-0.5	332.0	15.06	50.0	12.89	42.8

It is noted from table 5.16 that the quantity of TMC-H used correlated very well between CHIT-H and H-AA-0.5 when the weight of beads equivalent to 50 mg ibuprofen was calculated.

The total volume of each half-cell after the final additions was 7 ml while the total exposed tissue surface area was 1.78 cm^2. At 20, 40, 60, 80, 100 and 120 minutes after the addition of the microbeads, 250 µl aliquots were taken from the receiver compartment for HPLC analysis and replaced with an equivalent amount of fresh PBS. It was previously shown by Hattingh (2002:83) that the maximum period to perform transport studies was approximately 120 minutes before structural damage to the epithelium occurred. Experiments were performed in six-fold.

The average apparent permeability coefficient (P_{app}) was calculated according to the following equation (Grass and Sweetana, 1988:373):

$$P_{app} = \frac{dQ/dt}{60 \times A \times C_0} \quad (8)$$

where:

dQ/dt = the transport rate,

C_0 = the initial concentration of ibuprofen (100 %),

A = the area of exposed tissue (1.78 cm^2).

5.2.4 Statistical analysis

Statistical analysis of the experimental data was done to establish the degree of differences, if any, between the formulations tested. ANOVA (analysis of variation) was applied to the data using the Tukey's Studentised Range (HSD) Test from Statistica® 6 for Windows® (Microsoft® Corporation, Seattle, Washington, USA). The results were compared with each other and a p value of < 0.05 was tested. This indicated if statistically significant differences with a

confidence interval of 95 % ($p < 0.05$) were found between the formulations studied.

5.2.5 Results

The potential influence of TMC-H and ascorbic acid on the transport of ibuprofen from a chitosan microbead formulation was investigated in the basolateral/apical direction using Sweetana-Grass diffusion chambers. During the study chitosan microbeads containing 2 % (w/v) ibuprofen, 2 % (w/v) TMC-H and 0.5 % (w/v) ascorbic acid (H-AA-0.5) were compared with (1) a formulation containing only 2 % (w/v) ibuprofen and 2 % (w/v) TMC-H (CHIT-H) and (2) a plain ibuprofen loaded chitosan microbead (CHIT) as controls. The mean rate of ibuprofen transport (P_{app}) observed with the three formulations were compared. All experiments were performed in six-fold and the mean of the transport calculated for the six experiments was taken.

The P_{app} values of ibuprofen from the three different microbead formulations are presented in table 5.17 and the cumulative transport (%) is shown in figure 5.11. Although the H-AA-0.5 formulation exhibited the highest transport rate for ibuprofen, the P_{app} values obtained from the two formulations containing TMC-H (CHIT-H and H-AA-0.5) showed no significant difference in the transport rate of ibuprofen.

TABLE 5.17 The mean P_{app} values obtained for the transport of ibuprofen from CHIT, CHIT-H and H-AA-0.5 microbead formulations. Data is expressed as the mean ± SD (n=6).

Microbead formulation	P_{app} value (cm/s)
CHIT	$5.384 \pm 1.041 \times 10^{-7}$
CHIT-H	$7.631 \pm 1.475 \times 10^{-7}$
H-AA-0.5	$8.848 \pm 2.152 \times 10^{-7}$

Since the inclusion of ascorbic acid (0.5 % w/v) into the H-AA-0.5 formulation was the only variable between the CHIT-H and H-AA-0.5 formulations, it was concluded that the inclusion of ascorbic acid had no statistical significant effect on the transport rate of ibuprofen from the studied chitosan microbead formulations.

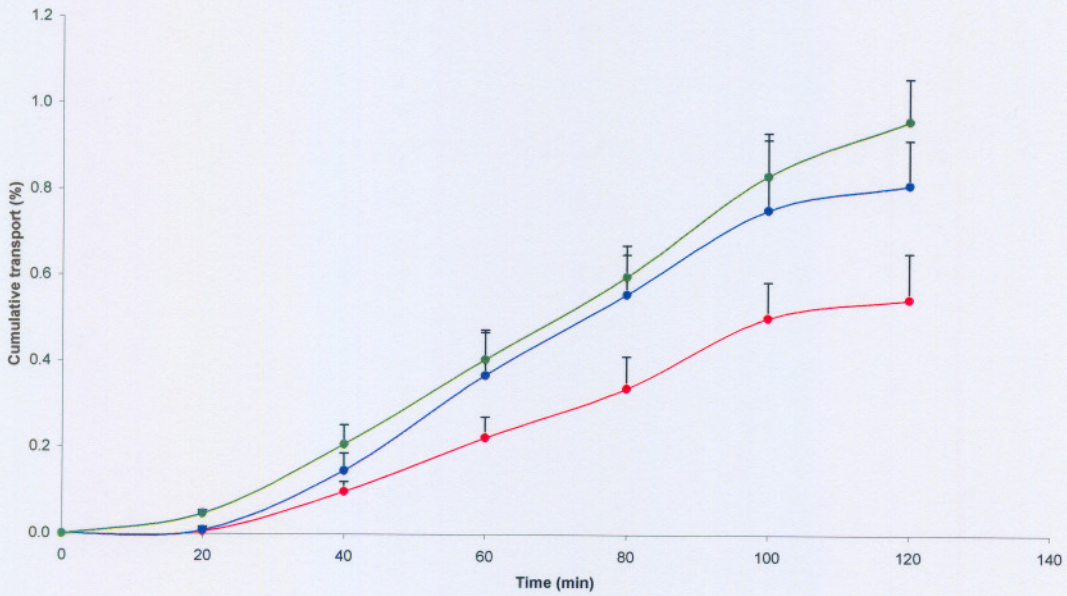


FIGURE 5.11 Cumulative transport (%) of ibuprofen from CHIT (●), CHIT-H (●) and H-AA-0.5 (●) microbeads. Data is expressed as the mean ± SD (n=6).

Compared to the CHIT formulation as control, both formulations containing TMC-H exhibited increased ibuprofen transport across *in vitro* rat jejunum. However, a statistical significant increase in transport was obtained only from the H-AA-0.5 formulation in comparison with the CHIT formulation. It is therefore concluded from the results obtained that the combination of TMC-H (2 % w/v) and ascorbic acid (0.5 % w/v) in chitosan microbeads lead to a statistical significant increase in the *in vitro* transport rate of ibuprofen through rat jejunum.

5.2.6 Conclusion

The successful release of TMC-H and ibuprofen from chitosan microbeads (solid dosage form) was shown in chapter 4. The inclusion of ascorbic acid further increased the release of TMC-H, probably due to an increased solubility of both TMC-H and chitosan. The *in vitro* permeation results obtained for ibuprofen in chapter 5 emphasised the absorption enhancing properties of TMC, since both formulations containing TMC-H (CHIT-H and H-AA-0.5) markedly increased the transport of ibuprofen in comparison with CHIT as control. Ibuprofen is a hydrophilic compound and is believed to be transported through the paracellular spaces, where opening of the tight junctions is proposed to be manipulated by TMC (Thanou *et al.*, 2000a:23; Kotzé *et al.*, 1997a:251). Although no statistical significant difference in the transport of ibuprofen were obtained between CHIT-H and H-AA-0.5, the formulation containing ascorbic acid significantly increased the transport of ibuprofen compared to the CHIT formulation as control.

Therefore it seems reasonable to assume that the inclusion of ascorbic acid into a chitosan microbead formulation containing TMC-H and ibuprofen, lead to a marked increase in TMC-H release. The increased availability of the TMC polymer at the site of transport significantly increased the permeation of ibuprofen through rat jejunum in comparison with chitosan alone.

SUMMARY AND FUTURE PROSPECTS

Chitosan has become well known as a multifunctional biopolymer with a wide range of applications both in the medical and pharmaceutical arena. The biotechnological applications of chitosan are based on the absorption enhancing and mucoadhesive capabilities for novel drug delivery systems.

As mucoadhesive polymer, chitosan attracted a lot of attention in the pharmaceutical community because of its ability to enhance the absorption of hydrophilic compounds across epithelial membranes. The absorption enhancing ability of chitosan is mediated by the positively charged amino groups in the structure of chitosan that induce interactions with the anionic sites on the cell membranes to alter tight junction integrity. However, in neutral and basic environments the chitosan molecule will lose its charge and precipitate from solution. Due to the lack of solubility at neutral and basic pH values this polymer is ineffective as absorption enhancer in certain parts of the gastrointestinal tract. *N*-trimethyl chitosan chloride (TMC) is a partially quaternised derivative of chitosan with superior solubility in a basic environment compared to other chitosan salts. The increase in solubility at higher pH values could be attributed to the replacement of the primary amino groups with quaternised amino groups (Domard *et al.*, 1986:105). Much has been said on the advantages of TMC, which retains both the absorption enhancing and mucoadhesive properties of the original chitosan (Hamman *et al.*, 2000:35; Kotzé *et al.*, 1997b:1197; Thanou *et al.*, 2000a:15).

Two TMC polymers with different degrees of quaternisation and different molecular weights were synthesised in a two-step reaction according to the method of Sieval *et al.* (1998:157) from high and medium molecular weight Chitoclear™ chitosan respectively. The synthesised TMC polymers were

characterised by using nuclear magnetic resonance (NMR) spectrometry, determination of the molecular weight by size exclusion chromatography connected to a multi-angle laser light scattering apparatus (SEC/MALLS), infrared spectrometry (IR) analysis and measurement of the intrinsic mucoadhesivity. These analytical techniques were used to determine differences in the molecular weight and the intrinsic mucoadhesivity of the various polymer solutions and to calculate the exact degree of quaternisation of each polymer. The degree of quaternisation calculated from the ^{1}H-NMR spectra for the medium molecular weight TMC (TMC-M) and the high molecular weight TMC (TMC-H) polymers were 74.7 % and 48.5 % respectively. The mean molecular weights of the synthesised TMC-M and TMC-H polymers were 64 100 g/mole and 233 700 g/mole respectively. IR spectra confirmed an increase in the degree of quaternisation of TMC-M which corresponded with the higher polymer chain length of lower quaternised TMC-H. A difference of 81.73 % between the intrinsic mucoadhesivity (IM) of TMC-M (144.23 %) and TMC-H (225.96 %) relative to pectin as control was calculated. This was expected as it is known that with an increase in the degree of quaternisation of TMC the mucoadhesive properties of the polymer are decreased (Snyman *et al.*, 2003:59). The difference of 125.96 % in the IM between TMC-H (225.96 %) and pectin (100.00 %) indicated that TMC-H exhibits relatively strong mucoadhesive properties.

Chitosan microbeads with a diameter of 800 to 1200 µm were prepared by ionotropic gelation as adapted from Bodmeier *et al.* (1989:1478). The microbeads contained either TMC-M or TMC-H, ibuprofen as model drug and different disintegrating agents.

Firstly, the effect of different concentrations TMC-M and TMC-H on chitosan microbeads was studied with results obtained from scanning electron microscopy (SEM), TMC loading capacity and microbead swelling behaviour. It was noticed from the SEM micrographs that the beads became less porous with a smoother outer-surface as the concentration of TMC increased, with TMC-H presenting denser beads compared to an equal concentration (w/v) TMC-M.

The more porous beads found with the 1 % (w/v) TMC-M and TMC-H also exhibited weak structural integrity. From the results obtained from morphology, percenatge drug loading capacity (% DLC) and swelling behaviour it was decided that a chitosan (3 % w/v) microbead containing 2 % (w/v) of either TMC-M or TMC-H will be used for further studies on the effects of different concentrations (0.1, 0.2 or 0.5 % w/v) additives (Ac-di-sol®, Explotab® or ascorbic acid).

After selection of the most suitable TMC concentration, the effect of varying concentration (0.1, 0.2 and 0.5 %) additives on TMC and ibuprofen release was studied. Commonly used modified cellulose gum (Ac-di-sol® (ADS)) and sodium starch glycolate (Explotab® (EXP)) were added as disintegrants to different microbead formulations to promote release of both the ibuprofen as model drug and TMC from the beads. It was also hypothesised that an increased acidity of the micro-environment in the beads might improve the solubility of both chitosan and TMC. A resultant increase in water-absorbing capacity and increased TMC and drug release from the microbeads might therefore follow. Thus, ascorbic acid (AA) was included to investigate the effect of acidity on the different microbead formulations. Scanning electron microscopy (SEM), TMC and ibuprofen loading capacity, microbead swelling behaviour and dissolution studies in phosphate buffered solution (PBS; pH 6.8) were performed on all microbead variants.

The morphological appearance of the synthesised microbead formulations corresponded well with each other and no significant differences were noted between the microbeads containing different TMC polymers and additives. All bead variants had a smooth outer surface with visible quantities of ibuprofen crystals entrapped in the chitosan gel structure. The dense inner matrix was found with all bead variants suggesting the loading of TMC, ibuprofen and additives into the crosslinked chitosan structure. The inclusion of different excipients in varying concentrations seemed to have little effect on the morphological integrity of the microbeads. The beads from the different

formulations were also rigid and exhibited favourable mechanical strength after lyophilisation.

It was noticed that the loading (% DLC) of TMC-M was much lower than that obtained with TMC-H while the inclusion of different additives in varying concentrations did not seem to have a profound influence on the loading of either TMC-M or TMC-H. The percentage drug content (% DC) of ibuprofen corresponded well between all the formulations except for the plain chitosan microbeads without any TMC or additives. The loading (% DLC) of ibuprofen was the highest in the plain chitosan microbeads since no TMC or additives competed with the ibuprofen to fill the inner cavities. All the formulations containing TMC swelled significantly more compared to the control, while no statistical significant ($p > 0.05$) differences were obtained in the percentage degree of swelling between the different formulations.

The fit factors (f_1 and f_2) were calculated according to a method by Moore and Flanner (1996:64) to describe the "closeness" of two compared dissolution profiles. It was noticed from the f_1 and f_2 values that the formulation containing TMC-H and 0.5 % (w/v) ascorbic acid was the only formulation with a significantly higher ibuprofen and TMC-H release profile compared to all other formulations tested. The chitosan microbead formulation containing 2 % (w/v) TMC-H and 0.5 % (w/v) ascorbic acid (H-AA-0.5) was thus used for the *in vitro* absorption studies through rat intestine. Chitosan containing only TMC-H (no ascorbic acid) (CHIT-H) and a plain chitosan microbead (CHIT) formulation was used as control formulations during the *in vitro* studies. The mean rate of ibuprofen transport (P_{app}) from the three different microbead formulations were found to be $5.384 \pm 1.041 \times 10^{-7}$ for CHIT, $7.631 \pm 1.475 \times 10^{-7}$ for CHIT-H and $8.848 \pm 2.152 \times 10^{-7}$ for H-AA-0.5. Although the H-AA-0.5 formulation exhibited the highest transport rate for ibuprofen, the P_{app} values obtained from the two formulations containing TMC-H (CHIT-H and H-AA-0.5) showed no significant difference in the transport rate of ibuprofen. Compared to the CHIT formulation as control, both formulations containing TMC-H exhibited increased ibuprofen transport across *in*

vitro rat jejunum. However, a statistical significant increase in transport was only obtained from the H-AA-0.5 formulation in comparison with the CHIT formulation.

It was therefore concluded from the results obtained that the combination of TMC-H (2 % w/v) and ascorbic acid (0.5 % w/v) in chitosan microbeads lead to a statistical significant increase in the *in vitro* transport rate of ibuprofen through rat jejunum.

Recommendations for future studies in this field are:

- The further reduction in the size of chitosan microbeads to obtain chitosan micro-particles and chitosan nano-particles.
- The use of different methods of preparation of chitosan microbeads in order to obtain different morphological changes.
- The inclusion of other pharmaceutical disintegrating agents into microbeads.
- The inclusion of TMC polymers with different degrees of quaternisation into chitosan microbeads.
- The inclusion of chitosan oligomers into chitosan microbeads.
- The inclusion of a hydrophilic model drug compound with lower aqueous solubility into microbeads.
- *In vivo* studies of chitosan microbeads containing TMC and a model drug compound.

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To my colleagues and friends from all departments at the School of Pharmacy (North-West University, Potchefstroom Campus), for their friendship, support and all the pleasurable hours we've spent together.

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ANNEXURE 1

Certificate of analysis

ChitoClear™

PRODUCT:	Fine grade chitosan made from fresh shrimp shells. Batch no: TD 012

CHEMICAL PARAMETERS:

DRY MATTER:	92.1%
ASH:	0.1 %
DEGREE OF DEACETYLATION	97.6% *(Titration method)*
SOLUBILITY:	> 99.9 % *(in 1 % acetic acid)*
TURBIDITY:	6 NTUs
VISCOSITY:	552 (mPa.s(cP)) *1 % solutions in 1 % acetic acid measured on a Brookfield RV DV-II viscometer, 25C, spindle no: RV2 at 60 rpm.*

FEATURE:

FLAKES:	0.1 - 6 mm

MICROBIOLOGICAL PARAMETERS:

TOTAL PLATE COUNT:	< 1 000 / g
ESCHERICHIA COLI:	absent
COLIFORME BACTERIA:	absent
SALMONELLA PR. 25 GRAM:	absent
MOULD AND YEAST:	< 100 / g

Primex Ingredients ASA

Christian Horst
Laboratory Manager

ANNEXURE 2

Certificate of analysis

ChitoClear™

PRODUCT:	Fine grade chitosan made from fresh shrimp shells. Batch no: TM 612

CHEMICAL PARAMETERS:

DRY MATTER:	95.7 %
ASH:	0.7 %
DEGREE OF DEACETYLATION	93.2 % *(Titration method)*
SOLUBILITY:	> 99.9 % *(in 1 % acetic acid)*
TURBIDITY:	9 NTUs
VISCOSITY:	12 (mPa.S(cp)) *1 % solutions in 1 % acetic acid measured on a Brookfield LVT viscometer, 25C, appropriate spindle At 30 rpm.*

FEATURE:

MICROMILLED POWDER:	100% finer than 60 mesh, 50% finer than 100 mesh
DENSITY:	0.46 g/cc *(USP method)*

MICROBIOLOGICAL PARAMETERS:

TOTAL PLATE COUNT:	< 1 000 / g
ESCHERICHIA COLI:	absent
COLIFORME BACTERIA:	absent
SALMONELLA PR. 25 GRAM:	absent
MOULD AND YEAST:	< 100 / g

Primex Ingredients ASA

Mette Aalstad Jansen
Laboratory Manager

ANNEXURE 3

FUJIAN SHIPBUILDING INDUSTRY GROUP CORP.

XIAMEN IMPORT AND EXPORT CO., LTD

4/F. NO 168,QIXING RD XIAMEN FUJIAN CHINA

TEL: 86-592-5117507 5117202 5117207 FAX:86-592-5117205

E-mail: shipie@public.xm.fj.cn

CERTIFICATE OF ANALYSIS FOR CHITOSAN

WCI 4219

Report No.: 000201

Batch No.: 000201	Quantity : 510kg	Report Date : Feb.03,2000
ITEMS TESTED		INSPECTION RESULTS
Appearance		Off white
Moisture		5.80%
Ash		0.83 %
Deacetylation		91.48%
Viscosity (0.5%)		20cp
Mesh		80mesh

L/C No.: IC04760069800102

*Drum's No.: 69--102

2000 -02- 29

ANNEXURE 4

Shasun Chemicals And Drugs Ltd.

IBUPROFEN EP(SN Grade)
CERTIFICATE OF ANALYSIS

Nature of Packing	: Sea Worthy Fibre Drum	Analytical Report No.	: FPIBU0202129
Sample Taken By	: Aru.Veera kumar	Batch Number	: IBU0202129
Date of Manufacture	: February 2002	Date of Analysis	: 27-02-2002
Expiry Date	: January 2007	Date of Report	: 27-02-2002
Batch Volume(Qty)	: 1500 kg.	Manufactured By	: **Shasun Chemicals And Drugs Limited,Pondicherry.**

S.No	TESTS	RESULTS	LIMITS
1.	Description	White crystalline powder	White crystalline powder
2.	Solubility	Complies	Freely soluble in Acetone,in Ether, in Methanol and in Methylene chloride. Dissolves in dilute solution of alkali hydroxides and carbonates. Practically insoluble in Water
3.	Clarity and colour of solution	Clear and colourless solution Obtained	10 % solution in Methanol should be clear and colourless
4.	Identification A) IR	Conforms	The IR spectrum of sample should be concordant with the spectrum of Ibuprofen Reference Standard
	B) UV	1.239 1.023	The ratio of absorbance at the max at 264 nm to that at 258 nm is 1.20 to 1.30 The ratio of absorbance at the max at 272 nm to that at 258 nm is 1.00 to 1.10
	C)Thin layer chromatography	Principal spot is similar in position, colour and size compared to Ibuprofen Reference Standard	Principal spot should be similar in position, colour and size compared to Ibuprofen Reference Standard
	D)Melting point	75.9 °C	75.0 TO 78.0 °C
5.	Optical rotation	0.000°	-0.050° TO +0.050°
6.	Heavy metals	LT 10 PPM	NMT 10 PPM
7.	Related substances (By HPLC) a). 2-(4-Isobutyryl Phenyl) Propionic Acid b). Any Unidentified impurity c). Total impurities	 0.022 % 0.024 % 0.126 %	 NMT 0.200 % NMT 0.100 % NMT 0.500 %
8.	Sulphated ash	0.042 %	NMT 0.100 %
9.	Loss on drying	0.089 %	NMT 0.500 %
10.	Assay (Dry basis)	99.82 %	NLT 98.50 % & NMT 101.00 %

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11.03.03

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ANNEXURE 4 (cont.)

Shasun Chemicals And Drugs Ltd.

IBUPROFEN EP(SN Grade)
CERTIFICATE OF ANALYSIS

Nature of Packing	: Sea Worthy Fibre Drum	Analytical Report No.	: FPIBU0202129
Sample Taken By	: Aru. Veera kumar	Batch Number	: IBU0202129
Date of Manufacture	: February 2002	Date of Analysis	: 27-02-2002
Expiry Date	: January 2007	Date of Report	: 27-02-2002
Batch Volume(Qty)	: 1500 kg.	Manufactured By	: **Shasun Chemicals And Drugs Limited,Pondicherry.**

S.No	TESTS	RESULTS	LIMITS
	ADDITIONAL TESTS		
a	Bulk Density (After 1250 Tappings)	0.705 g/mL	0.500 – 0.750 g/mL
b.	Particle Size	Complies	D90 MAXIMUM 300 MICRON
		Complies	D10 MINIMUM 20 MICRON
		Complies	D50 MINIMUM 60 MICRON
c.	Residual solvents		
	Methanol	N/A*	NMT 500 PPM
	Acetone	23.29 PPM	NMT 100 PPM
	Isopropyl Alcohol	LT 0.89 PPM	NMT 250 PPM
	Hexane	43.74 PPM	NMT 290 PPM
	Trichloro Ethylene	LT 0.19 PPM	NMT 80 PPM

OPINION: The Material Complies As Per **EP** Standard.

Note : NMT = Not more than NLT = Not less than LT = Less than N/A = Not applicable

*- METHANOL IS NOT USED IN IBUPROFEN (SN) SYNTHETIC PROCESS, TEST INCLUDED FOR COMPLIANCE WITH THE CERTIFICATE OF SUITABILITY

Analysed by	Checked by	Approved by:
Date : 27/02/2002	Date : 27/02/2002	Date : 27/02/2002
(S.Rajasudalaimuthu)	(K.Kumarasamy raja)	(N.Vinayagaperumal)
Senior Chemist	Senior Chemist	Dy.QC-Incharge

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ANNEXURE 5

SIGMA-ALDRICH

Certificate of Analysis

Product Name	Flufenamic acid
Product Number	F9005
Product Brand	SIGMA
CAS Number	530-78-9
Molecular Formula	$C_{14}H_{10}F_3NO_2$
Molecular Weight	281.23

TEST	SPECIFICATION LOT 113K1015 RESULTS
APPEARANCE	WHITE POWDER
SOLUBILITY	CLEAR COLORLESS SOLUTION AT 50 MG/ML IN ETHANOL
PURITY BY SODIUM HYDROXIDE TITRATION	100.0%
PURITY BY THIN LAYER CHROMATOGRAPHY	100%
QC ACCEPTANCE DATE	NOVEMBER 2003
PRODUCT CROSS REFERENCE INFORMATION	REPLACEMENT FOR ALDRICH #151300

Lori Schulz, Manager
Analytical Services
St. Louis, Missouri USA

ANNEXURE 6

YUNIBESITI YA BOKONE-BOPHIRIMA
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19 Oktober 2004

Prof J du Plessis
Bussie 36
Noordwes-Universiteit
(Potchefstroomkampus)

Geagte prof Du Plessis

GOEDKEURING VIR EKSPERIMENTERING MET DIERE

Hiermee wens ek u in kennis te stel dat u projek getiteld *"Establishment of the Sweetana-Grass diffusion model as an in vitro method to determine the permeability of drug substances for classifiaction purposes in the biopharmaceutical classification system (BCS)"* goedgekeur is met nommer 04D14.

Gebruik asseblief die nommer genoem in paragraaf 1 in alle korrespondensie rakende bogenoemde projek en let daarop dat daar van projekleiers verwag word om jaarliks in Junie aan die Etiekkomitee verslag te doen insake etiese aspekte van hulle projekte asook van publikasies wat daaruit voortgespruit het. U sal in Mei 2005 die dokumentasie hieroor ontvang.

Goedkeuring van die Etiekkomitee is vir 'n termyn van hoogstens 5 jaar geldig (volgens Senaatsbesluit van 4 November 1992, art 9.13.2). Vir die voortsetting van projekte na verstryking van hierdie tydperk moet opnuut goedkeuring verkry word.

Die Etiekkomitee wens u alle voorspoed met u werk toe.

Vriendelike groete

ESTELLE LE ROUX
NAMENS SEKRETARIAAT

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