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In-vitro and *In-vivo* Properties of a Lyophilised Nasal Dosage System

by

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Declaration

Dedication

I would like to dedicate this thesis to my parents for the countless things they have done for me, and to Kenny who gave me the constant encouragement and support that helped me through my studies.

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Abstract

The aim of this research was to examine the *in-vitro* and *in-vivo* properties of a lyophilised hydroxyproylmethylcellulose (HPMC) nasal insert dosage formulation, and assess its potential as a bioadhesive nasal formulation. *In-vitro* adhesive tests performed using a Texture Analyser gave variable results, prompting the development of novel dynamic adhesion and sliding adhesion tests designed to mimic the movement of the formulation over the nasal mucosa. Results indicated that increasing polymer molecular weight and concentration increased adhesion to a synthetic mucosal surface (agar), while over hydration resulted in a decrease in adhesion.

The use of a confocal laser scanning microscope (CLSM) to assess rate of water ingress into lyophilisates is described, with formulations showing an initial rapid rate of hydration, followed by a steady state rate of water ingress. Dynamic water vapour sorption studies demonstrated a high vapour sorption capacity, and a recrystallisation of amorphous mannitol, with scanning electron microscope (SEM) images depicting the effect of altering the relative mannitol and HPMC concentrations on the surface structure of the lyophilisates. SEM images also displayed the high internal porosity of the formulations, with pore size decreasing with increasing HPMC concentration, and showing correlation with rate of water ingress determined by the CLSM and an initial phase of linear insulin release demonstrated in dissolution studies.

In-vivo studies in sheep showed that the nasal insert increased bioavailability of nicotine over conventional spray and powder formulations, giving prolonged plasma nicotine levels. *In-vivo* nasal insulin absorption was not enhanced by the nasal insert, despite attempts to improve bioavailability by manipulating the formulation. A proposal for a study of the insert formulation in human volunteers is outlined.

In conclusion, the lyophilised nasal insert formulation displays adhesive properties influenced by polymer concentration, and increased nicotine bioavailability in sheep, but may not be suitable for nasal insulin administration.

Chapter 1 General Introduction

1.0 Introduction

The aim of this work was to fully investigate the *in-vitro* and *in-vivo* properties of a lyophilised nasal insert dosage form, the initial development of which has been previously described (Thapa et al., 1999d, Thapa et al., 1999c, Thapa et al., 1999b). Nasal delivery allows direct absorption of drugs across the nasal mucosa to the bloodstream, avoiding hepatic first-pass metabolism. A primary drawback to this route of administration is the rapid mucociliary transport rate of the nasal mucosa, allowing only a short window of opportunity for the absorption of drug. This may be problematic for the nasal absorption of drugs for which prolonged blood levels are desirable, or for high molecular weight proteins that may require extended nasal residence to promote absorption.

Hydroxypropylmethylcellulose (HPMC) is a polymer known to form a viscous gel, and the nasal insert (Figure 1.1) prepared from lyophilised HPMC solution offers both a method of nasal application which is more convenient to use than a gel, and theoretically increased nasal residence from the concentrated polymer gel which is expected to form upon hydration by the nasal mucosa.

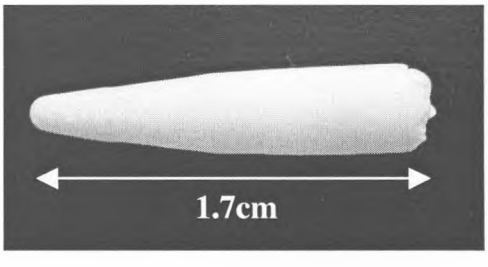


Figure 1.1 Lyophilised nasal insert.

In-vitro release profiles of nicotine hydrogen tartrate (NHT) have shown controlled release of NHT from such lyophilised formulations, and a preliminary *in-vivo* study of the nasal absorption of NHT suggested that the lyophilised nasal insert formulation exhibits extended nasal residence, resulting in enhanced and prolonged absorption of NHT, without the use of absorption promoters (Thapa et al., 2000).

This current study investigates the development of *in-vitro* adhesion tests to predict *in-vivo* performance, the influence of hydration and formulation variables on the behaviour of various lyophilised HPMC preparations, and the physical characteristics and behaviour of these formulations. *In-vivo* studies in sheep are then performed to evaluate the effect of formulation variables and drug properties on nasal absorption, and a proposal is made for an *in-vivo* study in human volunteers to evaluate the effect of formulation on nasal absorption of insulin, nasal residence (using scintigraphic imaging), and any correlation between the animal studies or *in-vitro* assessment.

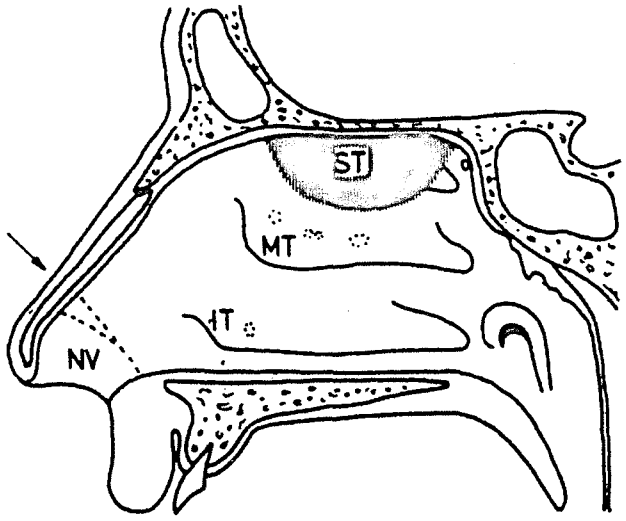
This chapter introduces a description of the anatomy of the nasal cavity, factors which are important in nasal absorption and bioadhesion, and methods which have been employed to examine adhesive formulations.

1.1 Nasal administration

Recently, nasal administration has increasingly been considered as an alternative route for drugs which cannot be delivered orally, and that require parenteral administration to achieve efficacy. The nasal route is useful as it provides a means of both avoiding the first pass metabolism effect, as well as potentially eliminating the need for often painful injections. In addition, the high perfusion rate of blood to the nasal cavity, combined with a large surface area (150 cm^2, Junginger, 1990) and only a very thin epithelial layer for a drug to cross (Quadir et al., 1999), means that it is ideally suited to drug delivery. The presence of many commercial preparations of drugs for nasal administration already available on the market displays the patient acceptability of this route of administration. In order to understand the obstacles to nasal delivery, it is necessary to consider the physiology of the human nose.

1.2 Nasal anatomy

The human nose consists of two nasal cavities, separated by the septal wall, the narrowest section of the nose being the nasal valve, near the front of the nose. The olfactory region, the area responsible for the detection of smell is situated in the posterior of the nose, near the top of the nasal cavity (Figure 1.2). Found in the posterior region of the nose are the inferior, middle and superior turbinates, which play an important role in the humidification and temperature regulation of inspired air, a prime function of the human nose. The high perfusion of arterial blood is responsible for the heating of inspired air. In one inspiration via the nose, room temperature air (23°C) at 40% humidity is conditioned to 32°C at 98% humidity (Mygind and Dahl, 1998), suited to conditions required by the lungs. Under normal conditions, respiration predominantly occurs via one side of the nose, with the other side becoming congested, a cycle which alternates every 3-7 hours (Jones, 2001).



Key:

Hatched area	olfactory region
NV	nasal vestibule
IT	inferior turbinate and orifice of the nasal lacrimal duct
MT	middle turbinate and orifices of frontal sinus, anterior ethmoidal sinuses and maxillary sinus
ST	superior turbinate and orifices of posterior ethmoidal sinuses

Figure 1.2 Structure of the nasal cavity

Reproduced from Mygind and Dahl, 1998.

The nasal mucosa is covered in ciliated columnar cells, except in the anterior part (about one third of the nose), which is unciliated (Mygind and Dahl, 1998). The columnar cells are also covered in microvilli, providing an ideal large surface area for absorption. Goblet cells exist in the nasal cavity, although they are thought to contribute little to the secretions in the nose compared to the submucosal glands (Mygind and Dahl, 1998), secretions from which form the mucus layer of the nose, which performs in conjunction with the cilia to trap foreign particles and remove them from the nose.

The mucus layer and any foreign particles are swept towards the back of the nasal cavity where they can be swallowed and subsequently destroyed in the gastro-intestinal tract. This forms part of the body's defence system, preventing substances such as bacteria or allergens from reaching the lungs (Ugwoke et al., 2001). It is therefore important that any nasal drug formulations do not have an irreversible effect on this process.

1.2.1 The mucus layer

Mucins or glycoproteins of varying molecular weights (100–10,000,000 daltons) constitute 3% of the mucus layer, with carbohydrates making up 80% of the weight of these mucins (Marttin et al., 1998). Water, containing proteins and electrolytes amongst other things, makes up the largest part of the mucous layer (90-95%) (Marttin et al., 1998).

The mucus layer is actually thought to be a double layer about 5-10μm thick (Mygind and Dahl, 1998, Ugwoke et al., 2001), with an upper slow moving viscous layer (gel layer Ugwoke et al., 2001) that is capable of trapping particles, and a lower layer (sol layer Ugwoke et al., 2001), less viscous and faster moving (Marttin et al., 1998), through which the cilia can beat with little resistance. The tips of the cilia just reach the bottom of the viscous layer and thereby propel this layer towards the nasopharynx. After a co-ordinated beat and full extension, the cilia can then move back to their original position easily through the watery layer below, in preparation for the next beat (Marttin et al., 1998).

The clearance half life of a particle trapped in the mucus layer is approximately 15-25 minutes. The rate of mucocilliary clearance varies between individuals and

can be altered by factors such as cigarette smoking, medicinal products and disease states such as allergic rhinitis, nasal polyps, cystic fibrosis or the common cold (Ugwoke et al., 2001, Jones, 2001). Any factor which alters the rate of mucocilliary clearance will have implications for nasal drug delivery, increasing or decreasing the time a drug is in contact with the mucosa for absorption for that patient.

1.3 Nasal absorption

A combination of the structure of the nasal mucosa as a barrier to absorption, and the properties of the drug itself will affect the extent to which a compound is absorbed nasally. Factors that may influence absorption include molecular weight, pH, solubility and the form in which the drug is administered, for example as drops, solution, suspension, spray, powder, or gel (Behl et al., 1998a). A further barrier to the absorption of insulin nasally may be degradation of the peptide by the enzymes present in the nasal cavity. Research has shown a high level of insulin degradation on exposure to rat nasal enzyme homogenates (Hirai et al., 1981b). This is unlikely to give an accurate reflection of behaviour *in-vivo*, as the rat nasal homogenate would contain enzymes from the cell, such as lysosomes, which might not ordinarily come into contact with the peptide.

Individual administration devices and technique can result in different sites of deposition within the nasal cavity, influencing absorption and clearance. Ideally, the drug would be deposited in the turbinate site for predictable absorption (Ugwoke et al., 2001). Potential for absorption directly to the central nervous system (CNS) is provided by formulations which can be administered directly to the olfactory region (Chou and Donovan, 1998, Illum, 2000), of interest for the administration of drugs for conditions such as Parkinson's disease (Kao et al., 2000), which tend to have a high incidence of side effects that may be reduced by the lower doses required for direct CNS delivery.

1.3.1 Hydrophilicity/lipophilicity

Generally, drugs which are in solution will be rapidly absorbed nasally (Hussain, 1998), although it appears that hydrophilic compounds are not readily absorbed above around 1000 daltons (Donovan and Huang, 1998, McMartin et al., 1987), as they are thought to be absorbed via aqueous channels in the nasal mucosa (Chien et al., 1989), and so absorption decreases as molecular weight increases.

The absorption of lipophilic drugs is thought to occur via an alternative pathway to aqueous channels, namely the transcellular route. The extent of absorption is linked to the drug's lipophilicity, and its partition coefficient between the nasal mucosa and any buffer solution used (Behl et al., 1998a, Corbo et al., 1998), allowing the drug to partition into the lipid cell membrane (Hinchcliffe and Illum, 1999).

1.3.2 The effect of pH

Linked to the theory of hydrophilicity and lipophilicity is the role that pH is thought to play in nasal drug absorption. The effect of pH can be variable, but can generally be linked to how the pH would affect the ionisation or structure of the molecule (Behl et al., 1998a, Dondeti et al., 1996). Nicotine (Figure 1.3), a basic alkaloid of pKa 8.0 (Schneider et al., 1996), showed a much higher absorption *in vitro* at pH 8.8, when it is unionised than at pH 2.0 (Chen et al., 1999).

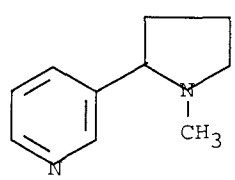


Figure 1.3 Chemical structure of nicotine.

In an *in vitro* study in Caco-2 monolayers involving chitosan as a promoting agent, decreasing pH gave an increase in the permeability of mannitol. This was thought to be due to the lower pH "uncoiling" the chitosan molecule and increasing ionisation density, resulting in better opportunity for close contact with the monolayer to exert a permeation enhancing effect (Tengamnuay et al., 2000). Midazolam absorption in rats was found to be dependant on a pH greater than 4, when it existed in at least 1% of its unionised form, due to the pKa of midazolam being 6.1 (Olivier et al., 2001). Some other increases of absorption at low pH are thought to be a result of damage to the nasal mucosa (Tengamnuay et al., 2000, Ohwaki et al., 1985). This effect of pH is therefore likely to influence the choice of any buffer solution used (Dondeti et al., 1996). Increasing the pH of scopolamine hydrobromide solutions was shown to increase nasal absorption in humans (Ahmed et al., 2000), although no potential explanation was given for this.

1.3.3 Osmolarity

Osmolarity has been reported to play a role in nasal absorption, although accounts are conflicting. One study (Ohwaki et al., 1987) found that hypertonic solutions promoted absorption of secretin in rats, thought to be a result of the observed shrinkage of cells in the mucosa, allowing more drug to permeate. It was found elsewhere that addition of osmotic agents to adhesive gels containing insulin resulted in further decreases in plasma glucose concentrations in rats (Pereswetoff-Morath and Edman, 1995). However, it was found in rats that only very hypoosmotic solutions led to significant absorption of midazolam, although pH may have played a role here too (Olivier et al., 2001). The authors suggested that a resultant swelling of the mucosa aided paracellular diffusion of midazolam.

The finding that hypertonic solutions promote absorption would appear to agree with the theory that some polymers promote absorption via uptake of water and subsequent shrinkage of epithelial cells, resulting in a widening of tight junctions between the cells as shown by (Edman et al., 1992). In some cases it is also proposed that bioadhesive polymers may further aid absorption of drugs via an enhancing action on the nasal mucosa itself.

1.3.4 Other factors

When a drug is administered as a solution the volume applied at one time is of consequence (Dondeti et al., 1996), and inherently restricted due to the size of the nasal cavity (Ugwoke et al., 2001). Optimal volumes appear to be between 50-100μL, with better results obtained by halving the dose and administering twice if the volume is too large (Dondeti et al., 1996). This effect was demonstrated in a study by (Harris et al., 1988a), who reported that administration of desmopressin as a 2 x 50μL dose gave significantly higher peak plasma and area under the curve (AUC) values than either a 1 x 50μL or 1 x 100μL dose. This study also found that an increase in desmopressin concentration gave rise to an increase in bioavailability.

The particle size of the drug can be important in terms of both its dissolution rate and surface area if administered as a powder, and also in terms of the fact that only those particles of 5-10 μm tend to remain in the nasal cavity when inhaled (Behl et al., 1998a). When administered as a solution it was found that desmopressin absorption was 2-3 times higher when formulated as a spray rather than drops (Harris et al., 1986), concluded to be due to the decreased clearance rate of the nasal spray.

1.4 Bioadhesion

Bioadhesive substances that will adhere to the nasal mucosa are often used in attempts to overcome rapid mucociliary clearance of the formulation from the nasal cavity. Bioadhesion has been defined as the ability of a material to adhere to a biological tissue for an extended period of time (Dondeti et al., 1996). The main purpose of a bioadhesive is to bind in some way with the mucous layer that covers the nasal epithelium, thereby decreasing its rate of clearance from the nasal cavity. The resultant increased contact time with the mucosa gives the potential to adapt the formulation for controlled release of the drug if desired (Alur et al., 1999), along with the opportunity for increased absorption (Dondeti et al., 1996). Bioadhesion is generally achieved with the use of polymers.

Factors that are thought to be of importance to the overall bioadhesion of a polymer system include functional groups, molecular weight, crosslinking, concentration and pH (Ugwoke et al., 2001). It is unclear which is the most

important, and it seems probable that most polymers exhibit bioadhesion due to a combination of effects, rather than as a result of any one mechanism in particular.

1.4.1 Swelling

The degree of swelling, or water absorption exhibited by a polymer is an important factor in bioadhesive behaviour (Dondeti et al., 1996, Alur et al., 1999, Nakamura et al., 1999, Bernkop-Schnurch and Gilge, 2000, Nakamura et al., 1996), as the polymer must have the ability to take up water from the mucosal surface (Illum et al., 1987). The degree of swelling appears to effect the extent of bioadhesion by forming a gel structure with increasing flexibility of the polymer chains (Alur et al., 1999), permitting intimate contact between the polymer and the mucosal surface by allowing the chain sections of the polymer to interact and become entangled with the glycoproteins of the mucus (Nakamura et al., 1999, Illum et al., 1987, Lee et al., 2000). This entanglement appears to have an important role in bioadhesion (Lee et al., 2000), altering the properties of the mucous layer and reducing the rate of mucociliary clearance of the formulation. Although it is generally expected that a polymer which swells rapidly will adhere to the mucous more readily (Dondeti et al., 1996), it has been found (Henriksen et al., 1996) that over-hydration can occur (in this case with carbopol 934P), with loss of adhesion. This idea has also been supported by others (Mortazavi, 1995, Smart et al., 1984).

The length of chains contained within the polymer can also influence adhesion. Cross linking can affect adhesion negatively, as a result of reducing the length of chain within the polymer available for interaction with the mucus (Ugwoke et al., 2001).

1.4.2 Hydrophilic groups

Polymers that are bioadhesives generally contain groups such as hydroxyl or carboxyl moieties capable of forming hydrogen bonds (Lee et al., 2000, Mortazavi, 1995, He et al., 1998). This promotes the absorption of water by the polymer to produce the swelling thought to be required for mucoadhesion. Hydrophilic groups are also thought to be important for the polymer to hydrogen bond with the glycoproteins present in the mucous layer (Nakamura et al., 1996, Illum et al., 1987). The importance of this effect is demonstrated by the fact that in general, carboxylic acid containing polymers tend to show stronger adhesion when the group is un-dissociated and will be able to form hydrogen bonds (Dondeti et al., 1996, Nakamura et al., 1999). A report on the use of liposomes for enhancement of nasal absorption found that in rats, positively charged liposomes showed greater adhesion than uncharged (or negatively charged) liposomes (Iwanaga et al., 2000). This was thought to be a result of electrostatic interaction between the liposomes and the surface of the mucosa.

1.4.3 Molecular weight

In a study by (Smart et al., 1984) it was found that to obtain greatest bioadhesion with sodium carboxymethylcellulose, a molecular weight of $\geq$ 78,600 daltons is desirable, and there is general agreement that adhesion increases with molecular weight (Dondeti et al., 1996, Lee et al., 2000).

There may be a plateau effect for some molecules however, as observations showed that dextrans with a molecular weight of 19,500,000 daltons were no more adhesive than those of 200,000 daltons (Ugwoke et al., 2001). It is suggested that the helical structure of the high molecular weight dextran resulted in binding sites being shielded inside the structure, making them unavailable for adhesion. Therefore the importance of the effect of molecular weight may vary depending on the conformation of the adhesive. Polymers with a linear structure will show increased adhesion with increasing molecular weight, as the high molecular weight translates into an increase in the chain length of the polymer, increasing entanglement with mucous glycoproteins (Dondeti et al., 1996).

1.4.4 Viscosity

Viscosity can also play a role in the extent of bioadhesion of the substance in question. It has been shown that viscous solutions resulted in an increased residence time of the polymer in the nasal cavity (Dondeti et al., 1995), although this is contradictory to the idea that close contact is required between the chain segments of the polymer and the mucus glycoprotein for improved bioadhesion, and that with a more viscous solution it may become more difficult to achieve this close contact (Dyvik and Graffner, 1992, Nagai et al., 1984). Increasing concentration of polymer would gradually result in a decrease in the mobility of the polymer chain, decreasing the extent of interaction with the mucus glycoprotein, resulting in a loss of bioadhesive efficacy (Dondeti et al., 1996, Lee et al., 2000). A result of this theory is the suggestion that cross-linking may reduce the mobility of polymer chains, and therefore decrease the opportunity for interaction with mucus, lowering adhesion (Lee et al., 2000).

1.4.5 Bonding

Recent work (Bernkop-Schnurch et al., 1999, Bernkop-Schnurch and Steininger, 2000) investigated the theory that as bioadhesive polymers often rely upon non-covalent bonds for interaction with the mucus glycoproteins, a substance capable of covalently bonding with the mucus would achieve even greater bioadhesion. Polycarbophil-cysteine conjugates were investigated in an attempt to form disulphide bonds with the mucus, bonds already seen in many biological systems (Bernkop-Schnurch and Steininger, 2000). Results appeared to show some positive effects although there is still much investigation to be done in this area.

Other factors which are also considered to be involved in the process of bioadhesion include Van der Waal's forces (Illum et al., 1987, Lee et al., 2000), "number and size of pores in the hydrated network" (Dondeti et al., 1996) and the particle size of the formulation being studied (Illum et al., 1987).

1.5 Clearance from the nasal cavity

The main aim of bioadhesives is to promote absorption of the drug and/or achieve prolonged absorption by overcoming the rapid clearance rate from the nasal mucosa. This means that rate of clearance is an important factor to assess, and various *in-vitro* and *in-vivo* methods of investigating this have been reported.

1.5.1 *In-vitro* (mechanical tests)

1.5.1.1 Texture analyser

The Texture Analyser (TA) apparatus has been employed as a means of studying the bioadhesivity of different polymers and formulations (Figure 1.4). The TA can be used to study qualities such as adhesiveness, cohesiveness, compressibility and syringeability (Jones et al., 1997, Jones et al., 2002). In terms of assessment of bioadhesion, potentially the most useful measurements that the TA performs are maximum force of detachment and work of adhesion. These are defined respectively as the maximum force required to detach the substance being analysed from another substance, and the area under the force/distance curve (Wong et al., 1999). The most common method of assessing these parameters involves attaching the test substance to the end of the TA probe, which is then lowered at a defined rate until the test substance comes into contact with the surface to which its adhesion is to be measured. The probe then holds this position with a defined force, for a defined period of time, withdrawing at a set rate, and measuring the force required to do so.

This was demonstrated recently in a study of various polymer films (Eouani et al., 2001), which were lowered onto a mucosal surface, and the peak force of detachment and area under the force/distance curve measured as an indication of bioadhesive strength. An order of adhesion of the polymers under investigation was determined using this method.

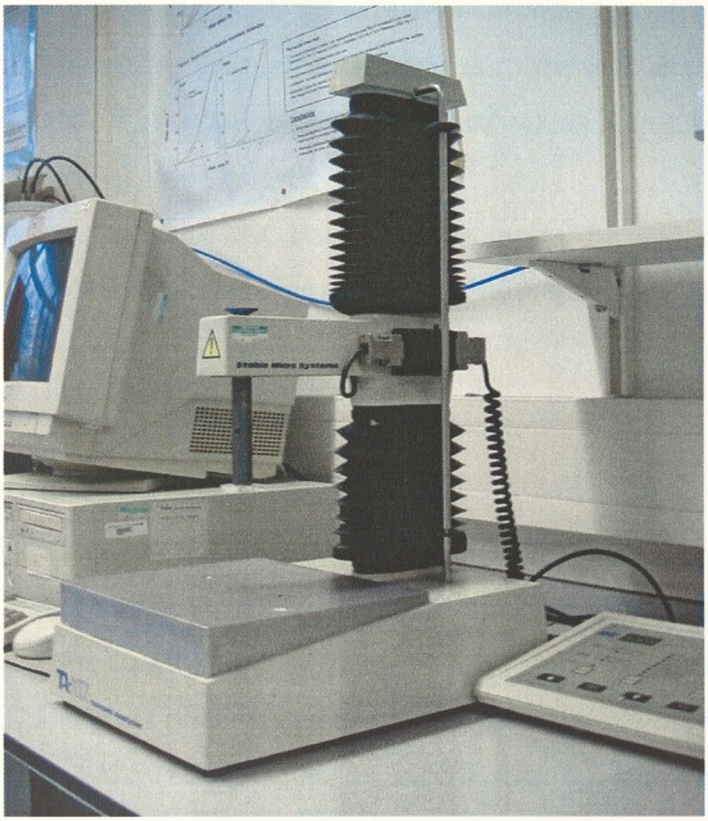


Figure 1.4 The Texture Analyser apparatus.

1.5.1.1.1 Instrument variables

Altering the TA instrument variables influences the outcome of adhesion tests, and it was found that contact time and rate of withdrawal parameters could have a significant effect on the results of the test. An increased contact time of Carbopol 974P tablets produced higher adhesion measurements, and the faster the rate of withdrawal of the probe, the higher the work of adhesion (Wong et al., 1999).

However, the effect of the contact force was seen to have a plateau effect on adhesion, and indeed too great an increase in the contact force may result in a detrimental effect if tissue is being used to study adhesion. The authors suggested that the results produced by an increasing contact time were due to the fact that the substance will have longer to hydrate and swell, a factor known to affect adhesion.

Similar effects were seen with regard to the time of contact in a study of adhesive gels (Jones et al., 1997), although an investigation into the effects of different variables of a tensile testing machine found that the time of contact was relatively unimportant, whereas it was the compression rate which appeared to affect the detachment values (Dyvik and Graffner, 1992).

These findings demonstrate the importance of assessing and maintaining the optimum parameters for the tests being carried out in order to correctly evaluate the bioadhesives being studied.

1.5.1.2 Wilhelmy plate

Another method used to test bioadhesion is the Wilhelmy Plate, employed as a mechanical method of measuring the force of detachment (Figure 1.5). This involves the use of a glass plate, coated in the bioadhesive and dipped into mucus of natural or synthetic origin. The glass plate is attached to a device capable of measuring force, and the platform on which the container of mucus rests can be moved upwards or downwards at a defined rate. Therefore the maximum force required to detach the bioadhesive coated glass plate from the mucus can be measured. This measurement is usually performed after allowing the test substance on the glass plate sufficient contact time with the mucus.

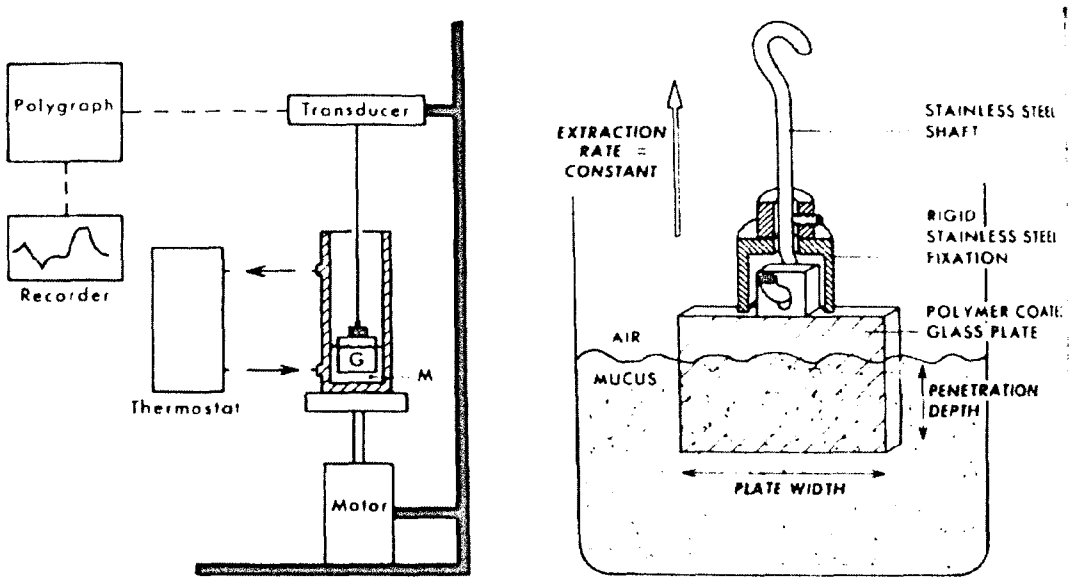


Figure 1.5 **Schematic representation of the Wilhelmy plate mucoadhesive force measurement apparatus and the device for the attachment of polymer coated glass plates.**

(G, glass plate, M, mucous gel), reproduced from Sam et al., 1992.

This experiment has been used to demonstrate that carboxymethylcellulose (CMC) gave a higher force of detachment than hydroxypropylmethylcellulose (HPMC) (Sam et al., 1992), and that both displayed much higher forces of detachment than for a glass plate alone. The method has also been used to study the effects of different variables, such as contact time and molecular weight, on the force of detachment (Smart et al., 1984).

1.5.1.3 Similar studies

An alternative method has been developed in which a disc of the polymer to be tested is attached to a weight, which is suspended from a balance (Smart, 1991). A section of tissue, immersed and secured in buffer solution, rests on a platform that can be moved vertically to achieve contact with the polymer disc. This was used to

show that Carbopol 934P (CP 934P) gave a significantly higher mean force of detachment than HPMC and interestingly, a much higher force of detachment than three commercial buccal formulations; Suscard Buccal, Buccastem and Adcortyl in Orabase. A similar method was reported by (Lehr et al., 1992) to have been used to investigate polycarbophil and chitosan.

The surface energy of the formulation may affect spreading over the mucosal surface and therefore adhesion. Analysis of surface energy has been used to predict adhesion of various polymers with apparent success, although no *in-vivo* measurements were made (Lehr et al., 1993). Predictions were compared with results previously reported in the literature and were found to match.

1.5.2 Ex-vivo

Freshly excised human turbinates have been used in a study of the transport rate of various chitosans (Aspden et al., 1997), where the chitosan was applied to the surface of the excised tissue, followed by a sprinkling of graphite particles. The movement of the graphite particles (and therefore the chitosan) was then tracked by computer imaging (Figure 1.6), and used to calculate the rate of transport of the bioadhesive chitosans.

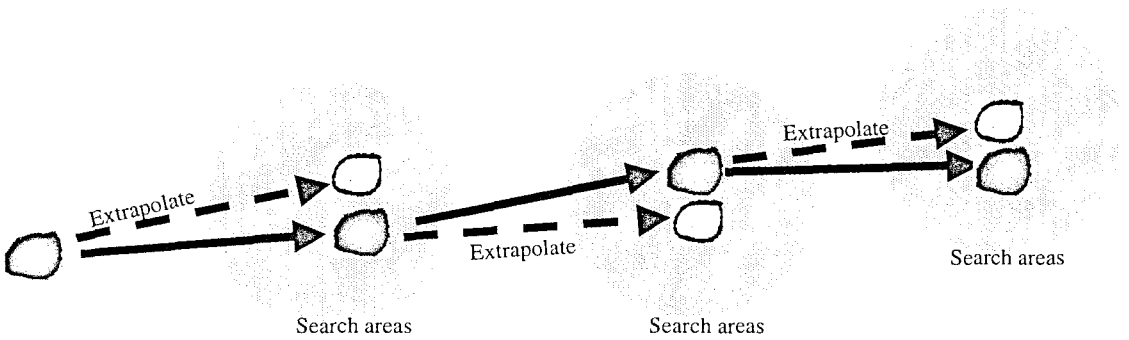


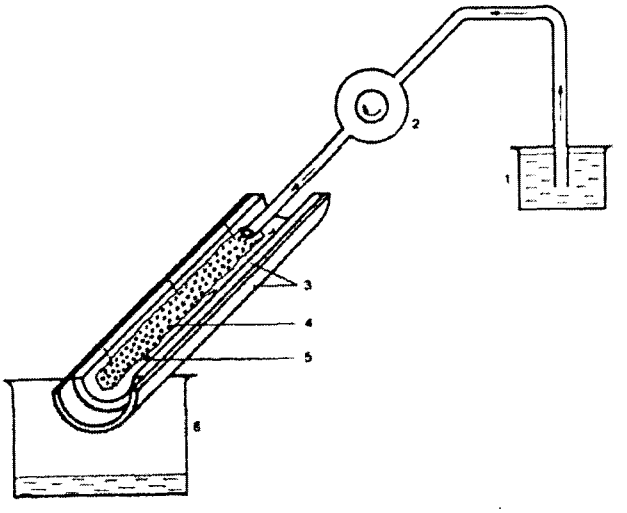
Figure 1.6 Schematic representation of particle tracking strategy.
Re-drawn from Aspden et al., 1995.

This test tracks the non linear movement of particles across the mucosa, rather than the usual procedure of assuming linear movement. However, in one study (Mason et al., 1995) it was shown that results from a transit time test involving saccharin in humans displayed no correlation with the particle tracking results in the excised turbinates of the same patients. This may have been a result of the different behaviour of the saccharin compared to graphite *in-vivo*, and differences in nasal anatomy of the patients.

A similar model used a frog palate as the biological substrate to study HA and HA/chitosan microspheres which were shown to be significantly more effective at reducing the mucociliary transport rate than chitosan microspheres (Lim et al., 2000). Another study using the frog palate method demonstrated that chitosans produced a transient decrease in the mucociliary transport velocity (Aspden et al., 1995).

A different approach using tissue was taken in a study (Rao and Buri, 1989) where researchers coated glass beads with the polymer being examined, and placed them on tissue which was held at an incline (Figure 1.7). Buffer solution or dilute hydrochloric acid (HCl) was then washed over the tissue, and any beads which did not adhere were collected, weighed, and used as a measure of the polymer coating's

bioadhesion. This study found the order of adhesivity of substances to stomach tissue to be PC > sodium carboxymethyl cellulose (Na CMC) > HPMC > pectin > methyl cellulose (MC). A similar investigation reported the use of a flow through system on porcine oesophageal tissue to assess the adhesion of fluorescently labelled alginate solutions (Batchelor et al., 2002).



Key:

1. Reservoir containing the washing solution
2. peristaltic pump
3. plastic support
4. tissue
5. pin
6. receiver for collecting the washings

Figure 1.7 Schematic diagram of the assembly used to test the bioadhesion of the microparticles.

Reproduced from Rao and Buri, 1989.

Clearance studies have also been used as a means to evaluate the toxicity of a substance that is being evaluated for nasal delivery. By measuring the initial transport rate or ciliary beat frequency, the ability of the mucosa to recover from the application of a substance can be evaluated according to the subsequent measures of transport rate. It is important that any effects on the transport rate are reversible, as this indicates that the mucosa has recovered from the assault. This has been used for investigation of cyclodextrins (Agu et al., 2000, Boek et al., 1999), buffer solutions (Aspden et al., 1995), various anaesthetic agents (Mayor and Illum, 1997), phosphatidylcholines (Gizurarson et al., 1990), bile salts (Gizurarson et al., 1990) and fusidate derivatives (Gizurarson et al., 1990, Boek et al., 1999).

1.5.3 *In-vivo*

Ultimately, *in-vivo* studies will provide the most realistic assessment of the rate of clearance. The use of Fluospheres has been employed (Zhou and Donovan, 1996), by mixing a defined quantity of Fluospheres with the bioadhesive gel and applying to the nasal cavity of rats. The clearance of the gel was then calculated by swabbing the oral cavity, and analysing the Fluosphere content of the swab using fluorescence spectrometry. This study found that 3% HPMC, 3% CMC, 3% MC, 3% chitosan G and 0.4% CP 934P gels all significantly decreased clearance compared with the control (assessed by quantifying the total mass recovered in 2 hours). This study found that it can be misleading to rely on one type of measurement for clearance, as the order of decrease in clearance (time for 90% clearance) was found to be 3% MC > 3% HPMC > 3% CMC > 3% chitosan G > 0.4% CP 934P, and yet the order of least mass recovered was 3%chitosan G < 3% HPMC < 3% MC < 3%CMC < 0.4% CP 934P.

In another study involving rats (Jung et al., 2000), the mean residence time of nicotine proliposomes was calculated by dividing the area under the moment curve by the area under the curve for nicotine plasma concentration. The mean residence time (MRT) for nicotine hydrogen tartrate (NHT) proliposomes and sorbitol mixture was significantly greater than for NHT solution alone (Table 1.1). This study

suggested that these formulations could overcome the problems of the short half-life of nicotine nasal sprays due to clearance from the nasal cavity.

Chitosan formulations previously shown to decrease mucociliary transport in studies using the frog palate method were then examined in humans (Aspden et al., 1997) using a test where saccharin is added to the substance applied nasally, and the time taken for the subject to taste the saccharin is taken as a measure of bioadhesion. This test showed no differences in any of the formulations after 1 hour, although it is relatively subjective.

This shows advantages and disadvantages of both *in-vivo* and *in-vitro* tests, *in-vivo* studies provide a real-life measurement, but as a result of patient variations cannot necessarily give a definitive value with which to compare formulations. The *in-vitro* test gives us the more accurate adhesion measurement from which to compare substances, but cannot represent the conditions which would apply to the substances within the nasal cavity.

Table 1.1 **Pharmacokinetic parameters (mean ± S.D., n=4) of nicotine following i.n. administration of nicotine in rats.**

Nicotine base (NB) and nicotine hydrogen tartrate (NS) administered at a nicotine dose of 1mg/Kg in the form of saline solution, proliposomes and a sorbitol mixture (NS/sorbitol, 1:9 v/v) in rats[a], from Jung et al., 2000.

	Nicotine base	Nicotine hydrogen tartrate
	Solutions/proliposomes	Solutions/proliposomes/sorbitol mixture
AUC (μg/ml/min)	65.8 ±27.9 / 56.1 ±34.7	27.8 ±7.12 / 39.0 ±12.8 / 30.7 ±2.30
MRT (min)	159.6 ±92.6 / 516.6 ±371.1	83.2 ±21.1 / 358.3 ±107.9 / 408.8 ±136.1[b]
$T_{1/2\beta}$ (min)	127.4 ±41.3 / 373.8 ±117.2	65.1 ±14.2 / 349.2 ±47.3 / 327.8 ±113.7[b]
C_{max} (ng/ml)	1748 ±153.5 / 395.1 ±243.2[b]	294.8 ±82.3 / 147.2 ±42.4 / 115.6 ±34.8[b]
T_{max} (min)	1.0 ±0.0 / 7.00 ±7.21	12.8 ±12.9 / 5.00 ±0.00 / 8.33 ±5.77
Bioavailability (%)	-/85.3	-/140.3/110.4

[a] Bioavailability was calculated against the corresponding AUC value following i.n. administration of saline solutions of corresponding compounds.

[b] Significantly different from corresponding solutions.

1.5.4 Gamma scintigraphy

A method which allows evaluation of mucoadhesion of test substances *in vivo*, providing an image of the distribution of the substance within the nose is gamma scintigraphy. This involves labelling the bioadhesive being investigated with the radioactive substance 99m-Technetium (^{99m}Tc), after which a gamma camera can be used to observe gamma emissions from the nose, showing the distribution and clearance of the bioadhesive. Using this method it has been demonstrated that solutions or powders (in this case chitosan) are cleared very quickly, usually with a half-life of around 15-21 minutes (Illum et al., 1987, Soane et al., 1999).

Carbopol 971P and CMC were shown to increase clearance times in rabbits, with percentage of carbopol 971P and CMC cleared after three hours being 12% and 27% respectively (Ugwoke et al., 2000a). This is compared with 58% for lactose powder.

1.5.4.1 Distribution within nasal cavity

Gamma scintigraphy has also been used to demonstrate that in spray formulations the administration technique does not appear to affect the distribution of a substance, while the volume used may (Newman et al., 1994). Another study (Suman et al., 1999) used scintigraphy to show that a modified nebuliser could achieve nasal deposition of solution in areas that a conventional pump spray could not reach (e.g. the olfactory region), resulting in an increased clearance time. The technique has also been used to demonstrate the superiority of a spray formulation in achieving uniform distribution over nasal drops (Bryant et al., 1999).

1.6 Absorption enhancers

The use of absorption enhancers in the formulation is another method commonly used to increase absorption of substances across the nasal mucosa. There have been many reports on the use of enhancers, many of which have shown positive results, although it is often unclear whether or not long term use will result in harmful side effects to the nasal mucosa.

1.6.1 Bile salts

Bile salts have often been investigated as absorption enhancers and are possibly the most widely used means of aiding absorption via use of a surfactant (Behl et al., 1998b). Typically used bile salts include cholate (CH), sodium taurocholate (ST), sodium glycocholate (SG) and sodium deoxycholate (SD) (Behl et al., 1998b, Junginger et al., 1999, Shao and Mitra, 1992, Bagger et al., 2001). The mechanisms by which bile salts are thought to promote absorption include increasing the permeability of the membrane, inhibiting proteolytic enzymes, formation of aqueous

pore pathways in the membrane or solubilisation of the drug in aqueous solution as a result of their surfactant properties (Behl et al., 1998b).

Preparations containing ST significantly increased insulin levels in diabetic rabbits, with or without the presence of MC (Dondeti et al., 1995), an effect which the authors suggested was due to the permeation enhancement of the ST. It has also been found (Shao and Mitra, 1992) that increasing the concentration of SG produced a linear increase in the absorption of aciclovir, and the same enhancer was found to produce a significant increase in melatonin absorption at a 1% concentration (Bechgaard et al., 1999). In another study (Junginger et al., 1999) 10μM SG produced a significant effect on the bioavailability of FD4 and buserelin.

1.6.2 Fusidate derivatives

Another commonly investigated group of absorption enhancers is the fusidate derivatives, such as sodium fusidate and sodium dihydrotaurofusidate (STDHF). These absorption enhancers are generally sodium salts of fusidic acid, an antibiotic compound. Fusidate derivatives show similar physical and chemical properties to the bile salts, and so have been suggested to enhance absorption in a similar manner (Behl et al., 1998b). STDHF has been shown to enhance nasal absorption of insulin (Shao and Mitra, 1992), and there have been numerous other studies which have shown that sodium taurodihydrofusidate, along with other fusidate derivatives enhances the absorption of various drugs (Behl et al., 1998b).

1.6.3 Chitosans

In addition to their bioadhesive properties, chitosans also appear to show absorption enhancing effects that are the result of a separate mechanism. It is thought that chitosans cause a transient opening in the tight junctions between the epithelial cells of the mucosa, thereby increasing the permeability of the mucosa to the drug in question (Aspden et al., 1996, Fernandez-Urrusuno et al., 1999). This may be a result of the cationic Ca^{2+}-binding properties of chitosans, 'trapping' the Ca^{2+} in the mucosa, as a correlation has been observed between Ca^{2+}-binding properties and octreotide absorption in rats (Oechslein et al., 1996).

There are a number of different chitosans available, of differing molecular weight and salt, and while they show slightly different enhancing activity, it is suggested that the difference between the individual chitosans is not significant (Aspden et al., 1996). As a general category of absorption enhancers however, chitosans have shown significant enhancement of insulin absorption (Aspden et al., 1996, Fernandez-Urrusuno et al., 1999, Illum et al., 1994a, Dyer et al., 2002) at concentrations of between 0.2-1%. Chitosan was also found to enhance the nasal absorption of the leutenising hormone releasing hormone (LHRH) – analogue goserelin in sheep (Illum et al., 2000). When goserelin was administered nasally with freeze dried chitosan microspheres a twenty-five fold increase in nasal absorption over simple goserelin solution was observed. This was suggested to be a result of a combination of the bioadhesive nature of chitosan resulting in an increased residence time in the nasal cavity, and the ability of the microspheres to take up large amounts of water resulting in an opening of tight cell junctions. The absorption enhancing properties of chitosan have also led to its investigation as a potential delivery system for nasal vaccines (Illum et al., 2001b).

Reports of a ceiling effect of about 0.1% concentration for promotion of absorption by chitosan (Dodane et al., 1999) appear to be completely contradicted by the finding in rats and sheep that suggests that greater enhancement of insulin absorption took place at concentrations of 0.2-0.5% and above (Illum et al., 1994a, Tengamnuay et al., 2000). An *in-vitro* study of release across caco-2 monolayers reported similar findings with a concentration dependant increase in mannitol permeability up to around 0.25% to 0.5% where a plateau effect was reached (Artursson et al., 1994).

1.6.4 Cyclodextrins

Cyclodextrins are cyclical oligomers of glucose, and are capable of forming "inclusion complexes" with drugs. The properties of the drug in question can be altered by this non-covalent 'inclusion', where the drug sits in lipophilic cavities in the cyclodextrin molecules (Behl et al., 1998b), effectively disguising the drugs own profile.

Dimethyl-β-cyclodextrin (DMβCD) has been shown to significantly improve nasal insulin absorption in rabbits at a 5% concentration (Schipper et al., 1993), although this effect was only seen with powder formulations, and not with solution where changes in insulin absorption observed were not significant. The observations made in this study were thought to be a result of interaction of DMβCD with lipids in the membrane. Also thought to be involved was the molecules' ability to prevent the formation of insulin hexamers, and to decrease the activity of proteolytic enzymes.

One study used DMβCD in combination with Dodecylmaloside, a non-ionic alkylglycoside, in an attempt to produce increased nasal absorption of insulin at lower concentrations of each enhancer (Ahsan et al., 2001). However, it was found that when combined, the two substances did not enhance absorption of insulin, thought to be a result of the two substances forming an inclusion complex with no absorption enhancing capabilities.

1.6.5 Phosphatidylcholines

Phospholipids investigated as absorption promoters include phosphatidylcholines such as lysophosphatidlycholine and didecanoyl-L-phosphatidylcholine (Behl et al., 1998b). Phosphatidylcholines are found in biological membranes and are surface active agents (Behl et al., 1998b). Changing the saturation and length of the hydrocarbon chain in the molecule (attached to a backbone of glycerol) will result in changes to the properties of the molecule (Behl et al., 1998b).

Lysophosphatidylcholine has been shown to have a significant enhancing effect on nasal absorption of a model drug in rats at 0.5% (Natsume et al., 1999), and in combination with degradable starch microspheres resulted in an increase in absorption of human growth hormone in sheep (Illum et al., 1990).

Nasally administered L-α-lyophosphatidylglycerol in combination with starch microspheres resulted in an absorption profile similar to that of a subcutaneous (s.c.) dose of G-CSF in sheep, where the intranasal dose was four times that of the s.c. (Gill et al., 1998). However, in terms of pharmacological response (stimulation of production of leucocytes and neutrophils) all formulations performed similarly. This was considered to be the result of there being a minimum level of circulating G-CSF required for stimulation.

1.6.6 Other absorption enhancers

Substances such as menthol (Shojaei et al., 1999), ammonium glycyrrhizinate and glycyrrhetinic acid (Dondeti et al., 1995), aminated gelatin (Wang et al., 2002), enzyme inhibitors (e.g. aminoboronic acid derivatives, amastatin) (Sarkar, 1992), microparticle resins (Takenaga et al., 1998), alkyl maltosides and alkyl sucrose esters (Pillion et al., 2002), glycofurol (Bechgaard et al., 1996), polyacrylic acid gel (Morimoto et al., 1985) and particulate carrier systems (e.g. alginic acid, microcrystalline cellulose, Oechslein et al., 1996) have also been investigated as absorption enhancers, with varying degrees of success.

Absorption enhancers have many other proposed mechanisms of action, such as causing pore formation in the membrane, lowering the membrane potential and increasing blood flow to the nose (Dondeti et al., 1996).

1.7 Membrane effects

One of the most important considerations in the administration of substances to the nose is that the substance will be safe, both as a one-off dose, as well as after long term repeated application. On studying nasal absorption of insulin, a drug which would require daily dosing, a group of researchers found that daily administration of bioadhesive materials reduced the bioavailability of insulin in rabbits (Callens et al., 2003). A nasal formulation that promotes drug absorption will be rendered useless if it causes damage to the nasal mucosa. In general, of most concern in terms of safety are the absorption promoters, although it is also important to consider the potential toxicity of any drug carrier, and indeed the drug itself.

Many absorption enhancers appear to act via an action on the nasal membrane, and as a result studies have been carried out to discover if these effects could be harmful. Different approaches have been taken, focussing on release of proteins from the membrane, histological studies and effects on the mucociliary clearance rate.

Chitosan has been extensively investigated, and has mostly been reported as being generally safe. After histological evaluation and enzyme release experiments one study concluded that chitosan caused "relatively mild and reversible effects" (Dodane et al., 1999) which is supported by other histological studies (Bernkop-Schnurch et al., 1999, Illum et al., 1994a, Tengamnuay et al., 2000) where recovery of the epithelium was such that it appeared similar to controls. In a study of protein and enzyme release it was concluded that "only negligible amounts of cell disruption and membrane disruption" occurred (Aspden et al., 1996), supported by the findings that free amine chitosans caused "minimal release" of a marker protein (Tengamnuay et al., 2000). Chitosan has also been found to have no subsequent effect on nasal clearance times in humans (Aspden et al., 1997). Other studies however have been less favourable (Carreno-Gomez and Duncan, 1997), and suggest that more research is required.

Other studies have investigated other absorption enhancers (Witschi and Mrsny, 1999, Dyvik and Graffner, 1992, Wang et al., 2002), and from results reported it is clear that many absorption enhancers will affect the nasal membrane in some way, meaning that great care must be taken when choosing an absorption enhancer.

1.8 Conclusion

The nasal route of administration for systemic absorption of drugs is very promising according to the results in the literature. The nasal cavity provides an absorption site which has the advantage of bypassing the first pass effect. The use of different formulation techniques (e.g. bioadhesive polymers) to enhance absorption may provide the opportunity to administer peptides via this route, thus avoiding painful injections. It must be remembered however that any formulation must not have long term damaging effects on the nasal mucosa.

Hypothesis

The hypothesis to be tested is that lyophilised HPMC nasal insert formulations can be used to achieve prolonged, enhanced nasal absorption of drug *in-vivo*.

Experimental

In order to test the hypothesis, the following investigations will be carried out:

1. *In-vitro* adhesion tests will be developed, in order to assess the effect formulation variables have on adhesion, and to attempt to create a model which can be used for rapid assessment of formulations as candidates for *in-vivo* examination.

2. Examination of the hydration process of the lyophilised formulations, and their affect on bioadhesivity.

3. Investigation of the physical properties of the lyophilised HPMC formulations, to assess factors which may be predictive of hydration and/or bioadhesive behaviour.

4. *In-vitro* release studies of drug in preparation for *in-vivo* investigations.

5. *In-vivo* nasal absorption studies in sheep of two model compounds with differing chemical properties, namely nicotine (as the hydrogen tartrate) and insulin, showing the effect of formulation parameters.

6. *In-vivo* nasal absorption study of insulin in human volunteers, with scintigraphic evaluation of nasal residence of the lyophilised HPMC nasal insert formulations, showing the effect of formulation parameters, and defining the relationship between nasal residence and pharmacokinetic profiles.

Chapter 2 Hydration Studies of Lyophilised Dosage Formulations

2.1 Introduction

The assessment of bioadhesion is often reported in the literature, with researchers describing various methods used in attempts to best compare formulations. Many assessments are based on tensile testing (Mortazavi and Smart, 1995), and the use of the Texture Analyser (Eouani et al., 2001, Jones et al., 1997) as first described by Tobyn et al (Tobyn et al., 1996). In these tests the force required to remove bioadhesive formulations from a substrate is used to quantify adhesive properties. The substrate employed can vary, perhaps according to availability and perceived suitability, ranging from an inert glass surface (Maggi et al., 1994), to *ex-vivo* mucosal substrates such as bovine buccal mucosa (Park and Munday, 2002, Maggi et al., 1994), and other substances such as tanned leather (Blanco-Fuente et al., 1996).

The exact method performed varies in a similar manner, with some researchers preferring to measure adhesion of pre-hydrated samples (Henriksen et al., 1996), others allowing only hydration from contact with the substrate (Hagerstrom and Edsman, 2001), some using only direct contact and others preferring to test in the presence of fluids such as mucins or phosphate buffered saline (PBS) (Lehr et al., 1992). Instrument variables such as contact time and force, and probe speed are often altered to assess their impact on adhesion (Wong et al., 1999).

The extent of variation in methods used to measure adhesion means that when considering the data presented on bioadhesion in the literature, the only real comparisons which can be made are between the order of adhesion found for different compounds in any single study.

Other tests have focussed on more dynamic processes such as particle tracking on an excised frog palate (Aspden et al., 1995), the use of gamma scintigraphy (Soane et al., 1999, Soane et al., 2001), or a system which involves washing the formulation in

question from a chosen substrate (Lehr et al., 1992, Batchelor et al., 2002, Rao and Buri, 1989).

An essential step in the bioadhesion process when considering lyophilised formulations will be the hydration of the formulation (Mortazavi and Smart, 1993) to allow the polymer chains to interact with the glycoproteins in the mucus layer. It is thought that an important part of the bioadhesive effect is a result of spreading of the hydrated polymer over the mucosa (Peppas and Buri, 1985), which in some way alters the nature of the mucus layer, reducing clearance rates. The hydration properties of bioadhesive formulations will therefore play an important part in the formation of a polymer gel and its mucoadhesive properties.

2.2 Materials and methods

2.2.1 Chemicals

HPMC powder (K4MP and K100LV grades) was obtained as a gift from Dow Chemicals (Michigan, USA). The first letter in the HPMC nomenclature represents the degree of substitution of the polymer, in this case the letter K denotes a methoxyl substitution of 22%, and a hydroxypropyl substitution of 8.1%. The number represents the viscosity in millipascal-seconds (mPa.s) of a 2% solution of that polymer in water at 20°C. M is used to represent 1000, LV means low viscosity and P refers to premium grade products.

D (-) mannitol powder (GPR), sodium hydroxide (NaOH) (GPR), polyethylene glycol (PEG), sucrose, lactose, polyvinylpyrrolidine (PVP), NaCMC, sodium chloride (NaCl) (GPR) and ultra pure agar were all purchased from BDH (VRW, Poole, UK). Nicotine hydrogen tartrate (NHT) and insulin (human recombinant expressed in Ecoli and bovine) were purchased from Sigma (Gillingham, UK). Nutrient agar was obtained from Microbiology, Department of Pharmaceutical Sciences, University of Strathclyde. Concentrated HCl (37% w/w) was from Allied Signal (Seelze, Germany). Cochineal dye was for culinary use.

2.2.2 Apparatus

A Texture Analyser (TA.XT2, Stable Micro Systems, UK) was used in adhesion mode with a 5Kg Load Cell, and data gathered were analysed using the Texture Expert software (Stable Micro Systems, UK). A Plexiglass disc attachment was manufactured in the Department of Pharmaceutical Sciences Workshop, University of Strathclyde, and was threaded in the centre of the disc to allow it to be screwed tightly onto the end of the TA probe.

Plastic petri dishes (Costar, 9cm diameter), six well culture plates (Costar 3.5cm diameter wells), agar culture plates (NUNC, 24.5 x 24.5cm), and glass sintered discs with a 20mm diameter were obtained from BDH (VRW Poole, UK). Vacuum formed blister packing cavities with wells of diameter 20mm and 12mm were

obtained as a gift and used as supplied by Quintiles (Livingston, UK). Polythene microcentrifuge tubes (0.2mL) were obtained from Life Sciences (Basingstoke, UK). A Virtis Advantage Freeze Drier, which carried out a cycle with preset stages, was used to lyophilise HPMC gels (Virtis, NY, USA).

A Bio-Rad MRC 1024ES (Bio-Rad Ltd., Hemel Hempstead, UK) Laser scanning Confocal imaging system, was used by scanning at 488nm using a Krypton/Argon light source.

2.2.3 Methods

2.2.3.1 Preparation of HPMC gels

HPMC gels were prepared by first dissolving the required amount of mannitol to produce a 1% w/w concentration in a small volume of distilled water (unless otherwise stated, all formulations contain 1% w/w mannitol). The appropriate weight of HPMC to produce the required final concentration was then added to the mixture, along with approximately one third of the final amount of distilled water (at 80-90°C). The mixture was stirred until a uniform solution was obtained, following which the remaining water was added. The solution was stirred until a uniform gel was obtained. The resultant gel was stored at 4°C overnight to remove air.

HPMC gels containing NHT were prepared by adding the required quantity of NHT equivalent to 2mg nicotine base per insert at the time of dissolving the mannitol in distilled water, and then continuing as described above.

HPMC gels containing insulin were prepared by dissolving the required quantity of insulin to produce 49iu per insert in 0.01M HCl, and adjusting pH as required with dropwise addition of 0.1M NaOH. The gel was then prepared as described above.

2.2.3.2 Preparation of lyophilised formulations

HPMC gels were lyophilised using the Virtis Advantage Freeze Drier, using the programme outlined in Table 2.1. Lyophilised discs were prepared by means of lyophilising the gel in the six well plates or blister packing cavities, and nasal inserts were prepared by lyophilising HPMC gel in polythene microcentrifuge tubes. All lyophilised formulations were transferred to a desiccator at room temperature immediately following completion of freeze drying, with the exception of insulin containing lyophilisates, which were stored under desiccation at 4°C. Samples were only removed immediately prior to experimentation.

Table 2.1 Freeze Drier Cycle.

Thermal Treatment

	Temp (°C)	Time (minutes)	R/H
Step 1	-30	60	H
Step 2	-40	120	H
Step 3	-50	120	H
Step 4	-60	120	H

Freeze	-30
Extra Freeze	60
Condenser	-40
Vacuum	200

	Primary Drying			
	Temp (°C)	**Time (minutes)**	**Vacuum (Millitorre)**	**R/H**
Step 1	10	300	100	H
Step 2	15	300	40	H
Step 3	20	180	40	H
Step 4	22	300	40	H

Post Heat	25	10	10

2.2.3.3 Texture Analyser adhesion tests

The TA was used in adhesion mode with a 5Kg Load Cell, and the TA cylindrical probe was modified for adhesion experiments by the addition of the Plexiglass disc to the end of the probe. The TA held the formulation in contact with an agar surface (intended to represent a synthetic nasal mucosal model, with water available for hydration) at a set force for the required period of time, and then measured the force required to remove the test substance from the surface of the agar.

2.2.3.3.1 Effect of K4MP concentration on adhesion

Adhesion of lyophilised K4MP formulations containing 0.5-3% K4MP was assessed using the TA with Plexiglass disc attachment. Nutrient agar was used as supplied, warmed to just above 50°C in a water bath and 20mL was cast into 7.5 cm diameter petri dishes. The agar was allowed to cool to room temperature, and sealed to avoid dehydration until use approximately 18 hours later.

The cone shape of the lyophilised nasal insert (Figure 2.1) means that the section of the lyophilisate to be tested would not provide a flat, even contact area for adhesive evaluation, which may lead to inconsistencies in results.

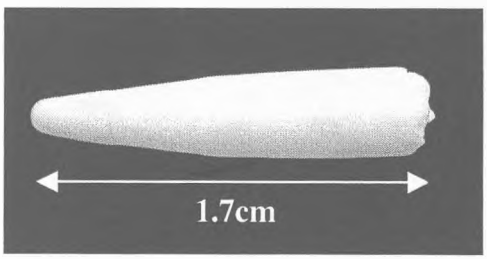


Figure 2.1 Cone shape of the lyophilised nasal insert.

In order to eliminate the shape of the nasal insert as a source of variability from the experiments HPMC formulations were tested in a disc shape, which would ensure that a defined surface area would be exposed to the agar, standardising experiments. K4MP gel (4mL) was measured into the wells of a six well plate and lyophilised as described in section 2.2.3.2.

The resultant lyophilised discs were removed from the wells and attached to the bottom of the Plexiglass disc on the TA probe using double sided tape. The TA probe was then lowered until the lyophilisate came into contact with the surface of the agar, and the formulation was held in place with a force of 2N for 30 seconds. The TA probe was then withdrawn and force and work of adhesion was measured. The TA parameters used are shown in Table 2.2.

Table 2.2 TA.XT2 Settings (1).

Option	Adhesive Test
Pre-test Speed	1mm/s
Test Speed	1mm/s
Post-test speed	1mm/s
Force	2N
Distance	10mm
Contact Time	30s
Trigger	Auto - 2N

2.2.3.3.2 Textural analysis of K4MP gels

The TA was used to analyse the adhesion of K4MP gels (0.25%, 0.5%, 0.75%, 1%, 1.25%. 1.5%, 1.75%, 2% K4MP with 1%mannitol) to the surface of Ultrapure agar (immediately and after 75 minutes of hydration), to determine effect of K4MP gel concentration, and to assess if the hydrated lyophilised formulations offered any increase in adhesion over the original gel component. Ultrapure agar (12g/L) was prepared by dissolving an appropriate amount of agar granules in distilled water, heated to approximately 90°C. The agar was then cast into petri dishes as described in section 2.2.3.3.1. Ultrapure agar was used in place of nutrient agar, to minimise any variation in the surface of the synthetic mucosal model.

The TA modified with Plexiglass attachment was used with the settings described in Table 2.3. K4MP gel (100μL) was measured onto the surface of the agar and the TA probe lowered onto the gel. The force applied by the TA probe was reduced from 2N to 0.5N to preserve the agar surface.

Table 2.3 TA.XT2 Settings (2).

Option	Adhesive Test
Pre-test Speed	1mm/s
Test Speed	1mm/s
Post-test speed	1mm/s
Force	0.5N
Distance	10mm
Contact Time	60s
Trigger	Auto – 0.5N

2.2.3.3.3 Elimination of further variables from TA measurements

Lyophilised discs consisting of 1.5 or 2mL 2% K4MP, prepared in six well plates, were placed on the surface of a 7.5cm diameter culture plate containing 12g/L Ultrapure agar and allowed to hydrate for 30 minutes. The TA probe modified with Plexiglass attachment was then lowered into the hydrated disc, and adhesion to the agar measured using parameters described in Table 2.3. The method of placing the lyophilisates directly onto the agar surface allowing hydration was designed to eliminate variation from undesirable measurement of adhesive forces between the lyophilisate, double sided tape and Plexiglass attachment.

2.2.3.3.4 Influence of extent of hydration and molecular weight

Lyophilised discs were prepared from 1.5mL of gels containing 1-2% K4MP or K100LV, lyophilised in 20mm diameter blister packing cavities. The discs were applied to the agar surface with a force of 5g for one minute, and then allowed to hydrate for 15, 30, 45, 60, 90, 20, 150, 180, 240, 300 and 360 minutes. The adhesion was measured by the TA as described in section 2.2.3.2, to determine how the extent of hydration influences adhesive properties.

2.2.3.4 Dynamic Adhesion method

Due to variability from adhesion measurements which were obtained from the TA, a test was designed which enabled assessment of adhesion under the dynamic conditions which would act on a formulation following administration to the nasal cavity. In the TA adhesion test the TA probe measured the force which was required to pull the substrate away at an angle perpendicular to the surface of the agar (Figure 2.2). When a formulation is administered to the nasal cavity and adheres to the mucosa, the force acting upon it will be the action of the cilia beating, moving the foreign object towards the anterior of the nose, in an attempt to remove it. The force acting on the nasal dose is that of a sliding action, parallel to the mucosal surface (Figure 2.3).

Therefore, a dynamic adhesion test was designed in order to mimic the simultaneous hydration, sliding and spreading processes which are expected to occur within the nasal cavity. Discs of lyophilised gel (0.6mL), stained with cochineal dye for clarity, were prepared in 12mm blister strips as described in section 2.2.3.2. Ultrapure agar (12g/L) was prepared as described in section 2.2.3.3.2, and 250mL was cast into 25cm x 25cm agar plates. The lyophilised discs were placed near the top of the plate and held in contact with the surface of the agar with a force of 0.05N for 60 seconds. The plate was then tilted to an 80° angle, allowing the hydrating discs to slide downwards, parallel to the surface (Figure 2.4). The distance travelled by a disc over a known time period was measured.

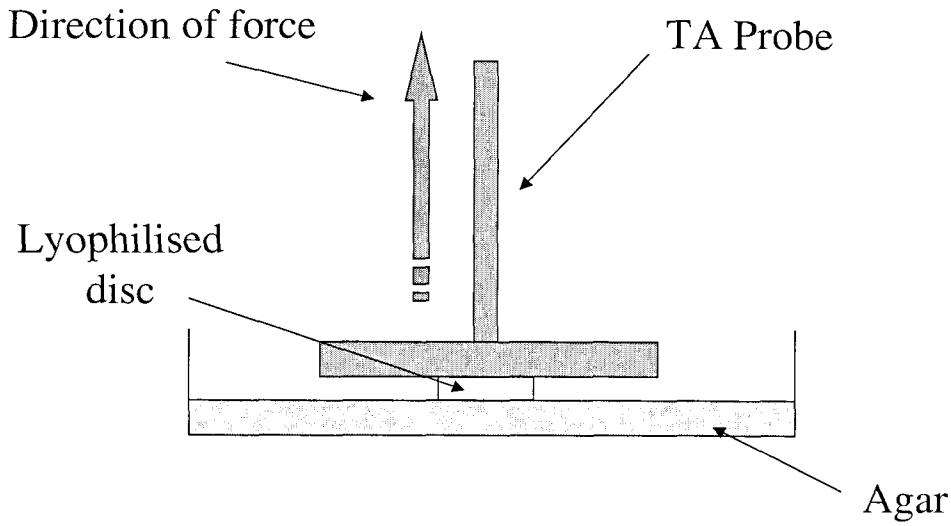


Figure 2.2 Texture Analyser action on lyophilised disc.

The direction of force applied is perpendicular to the synthetic mucosal surface.

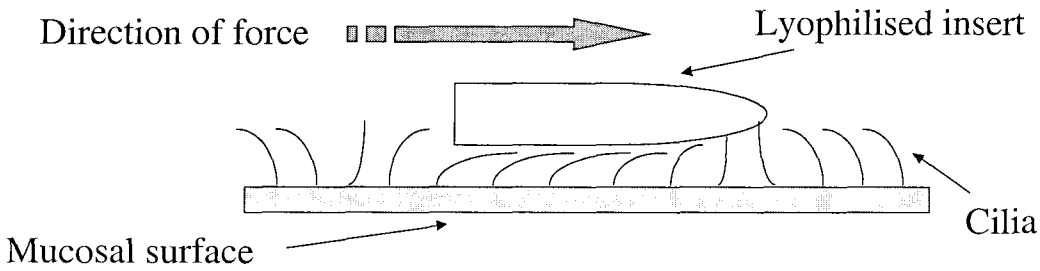


Figure 2.3 Action of nasal cilia on the lyophilised formulation in the nasal cavity.

The direction of force applied is parallel to the mucosal surface.

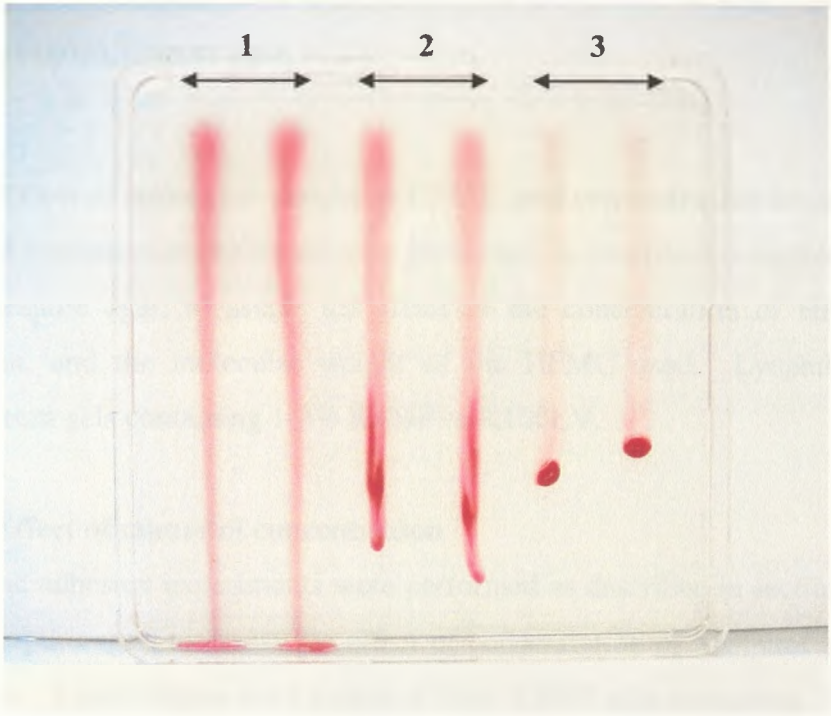


Figure 2.4 **Dynamic adhesion experiment showing differing hydration and adhesion between formulations.**

1 1% HPMC

2 2% HPMC

3 3% HPMC

2.2.3.4.1 Addition of dye to lyophilisates

HPMC gels were prepared, both with and without the addition of the cochineal food dye, in order to determine if the introduction of the food dye to the formulation affects adhesion measurements in any way. Gels were prepared with 1-3% K4MP and 0.5 or 2% mannitol. The experiment was performed as described in section 2.2.3.4 on 14g/L Ultrapure agar.

2.2.3.4.2 Effect of molecular weight of HPMC and concentration on adhesion

Dynamic adhesion experiments were performed as described in section 2.2.3.4 on 12g/L Ultrapure agar, to assess the effect of the concentration of HPMC in the formulation, and the molecular weight of the HPMC used. Lyophilisates were prepared from gels containing 1-3% K4MP or K100LV.

2.2.3.4.3 Effect of mannitol concentration

Dynamic adhesion experiments were performed as described in section 2.2.3.4 on 12g/L Ultrapure agar, to assess the effect of concentration of mannitol added to the formulation. Lyophilisates were prepared from K4MP gels containing 1-3% K4MP and 0.5-3% mannitol.

2.2.3.4.4 Effect of excipients

The effect of the addition of various excipients in place of mannitol was assessed. Substances which would be expected to enhance water uptake into the lyophilisate, NaCl, PEG, lactose and sucrose, were incorporated at different concentrations. Polymeric excipients were also incorporated, to assess if adhesion was increased by their addition. PVP and NaCMC were included to assess their influence on the formulation.

2.2.3.4.5 Addition of NHT or insulin

The effect of the addition of 2mg/0.26mL NHT, or 49iu/0.26mL bovine insulin to a 2% K4MP formulation was assessed using the Dynamic Adhesion test.

2.2.3.5 Simple hydration test

A simple hydration test assessing extent of water uptake by nasal inserts (0.5-3% K4MP) from the agar surface during experimental procedures was carried out. The inserts were weighed and placed on an agar surface (12g/L Ultrapure agar), removed after a defined length of time, re-weighed and water uptake calculated.

2.2.3.6 Texture analyser sliding adhesion test

A device was designed to create lyophilised K4MP discs which could be permitted to hydrate and form an adhesive gel on the surface of the agar, while retaining a portion which would be rigid enough to enable the formulation to be pulled along the surface of the agar, allowing the TA probe to measure the force required to do this. A frame for lyophilisation of K4MP discs was manufactured in the Department of Pharmaceutical Sciences workshop, University of Strathclyde. Plexiglas was used to form a hollow cup-like container (internal diameter of 20mm), with a portion of the base removed leaving only a thin lip on which the glass sinter could rest (Figure 2.5). Parafilm was stretched across the bottom of the container to avoid leakage of the gel from the underside. HPMC gel was then added to the device, and allowed to penetrate into the pores of the sinter before lyophilisation. The opening at the bottom of the device allowed the sinter/lyophilisate combination to be pushed out from underneath without damaging the shape of the freeze dried HPMC.

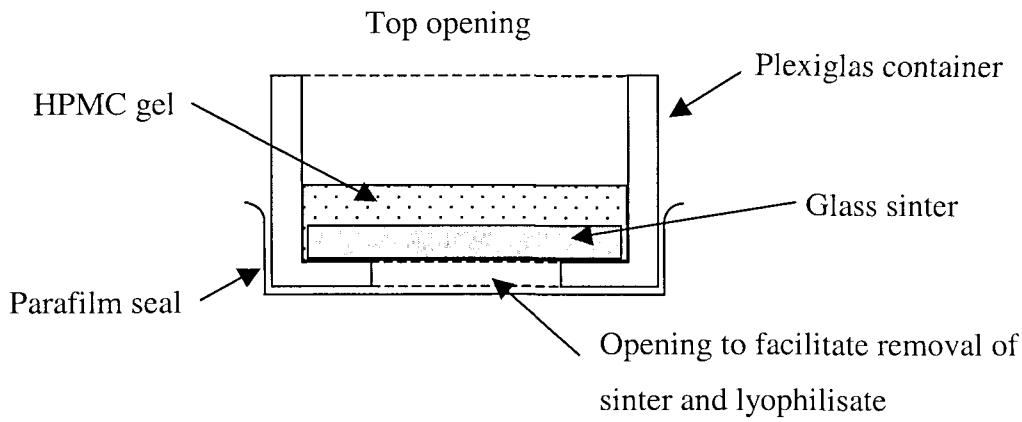


Figure 2.5 The Plexiglass device, and glass sinter resting within.

The TA was used in 'Measure Force in Tension' mode with a 5Kg Load Cell to quantify the force required to slide hydrated K4MP discs parallel to the surface of the synthetic mucosal model. A modified version of the TA Peel Strength Rig was used as shown in Figure 2.6.

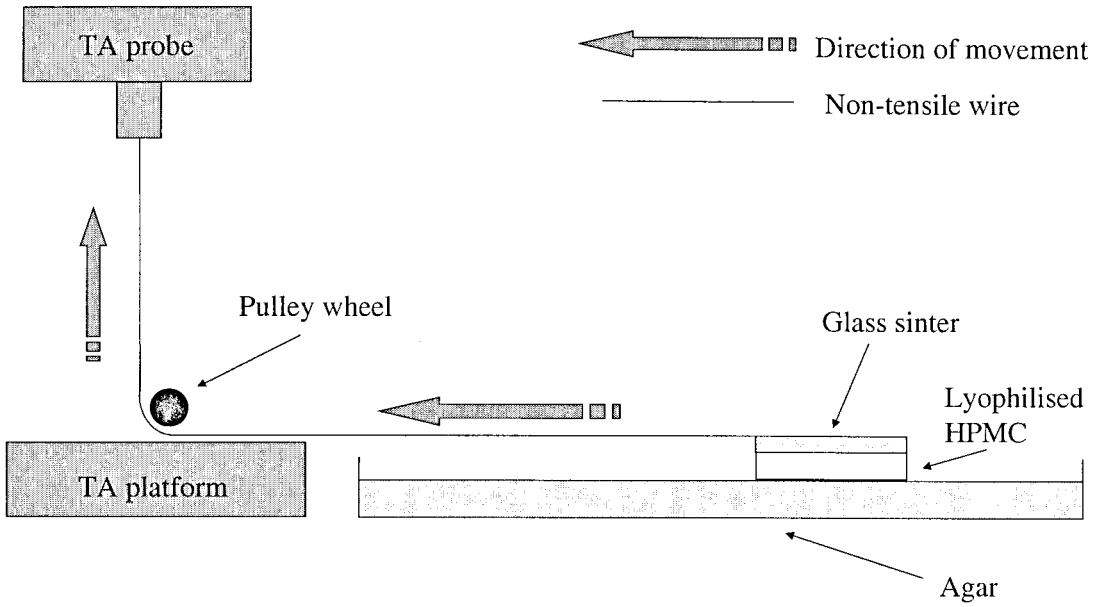


Figure 2.6 Texture Analyser apparatus, modified for sliding adhesion experiments.

K4MP gels were prepared as described in section 2.2.3.1. Lyophilised discs for Textural Analysis were then prepared by measuring 2mL of the K4MP gel (1-3%) onto the glass sintered disc (previously positioned within the Plexiglass container), and allowing the K4MP gel to penetrate through the sinter. The containers were then transferred to the freeze drier, which carried out a cycle with preset stages as previously described in section 2.2.3.2. Upon completion of lyophilisation, the glass sinter and lyophilisate combination were removed from the container by pressing out the sinter from the hole in the underside. In this way a unit was formed with lyophilised K4MP integrated with the glass sinter, and a portion protruding from the surface.

The discs were placed on the synthetic mucosal surface (12g/L Ultrapure agar) of an agar plate (25cm x 25cm), with a force of 5g for one minute. The lyophilised discs were then allowed to hydrate for a defined length of time, following which the

TA was used to measure the force required to pull the formulation parallel to the surface of the agar. The TA settings used are described in Table 2.4.

Table 2.4 TA.XT2 Settings (3).

Option	Measure Force in Tension
Pre-test Speed	3mm/s
Test Speed	1mm/s or 2mm/s
Post-test Speed	3mm/s
Max. Time	50s
Max. Distance	50mm
Trigger Force	Auto - 0.001N

2.2.3.7 Confocal laser scanning microscopy

The Confocal Laser Scanning Microscope (CLSM) enables a view to be obtained from inside the sample, on different points of the z-plane, in addition to the conventional x and y dimensions. Traditional microscopes capture images from a static light source located behind or in front of the sample, which can result in a lack of definition in the image. In a confocal system (Figure 2.7), the light source and the scanning beam are focussed on the same point, which enables a high resolution image from within the sample to be obtained, with no blur from out of focus images. This is because light from outwith the focal region does not reach the detector and appears black on the image.

Disc shaped lyophilisates were prepared from 0.6mL of K4MP gel as described in section 2.2.3.1 and 2.2.3.2, except with the addition of approximately 1mL of 0.6g/L sodium fluoroscein to the gel before lyophilisation.

Lyophilised discs were placed and compressed in a device designed to ensure consistent depth of sample for microscopy (Figure 2.8).

The top glass slide was then replaced with a cover slip. The device enabled the compartment containing the sample to be flooded with a weak water/rhodamine, ensuring that the disc was completely surrounded with solution available for rehydration (Figure 2.9).

Radial ingress of water into the sample was analysed using a Bio-Rad MRC 1024ES (Bio-Rad Ltd., Hemel Hempstead, UK) Laser Confocal Scanning imaging system coupled to a Nikon upright microscope using the argon laser line at 488nm. Scans were performed using 10x 0.25NA objective, at one or two second intervals over an area 1400μm x 1400μm. Scans were performed during initial hydration at the edge of the lyophilisate, and at a point closer to the centre of the disc when an apparent steady rate of hydration had been reached.

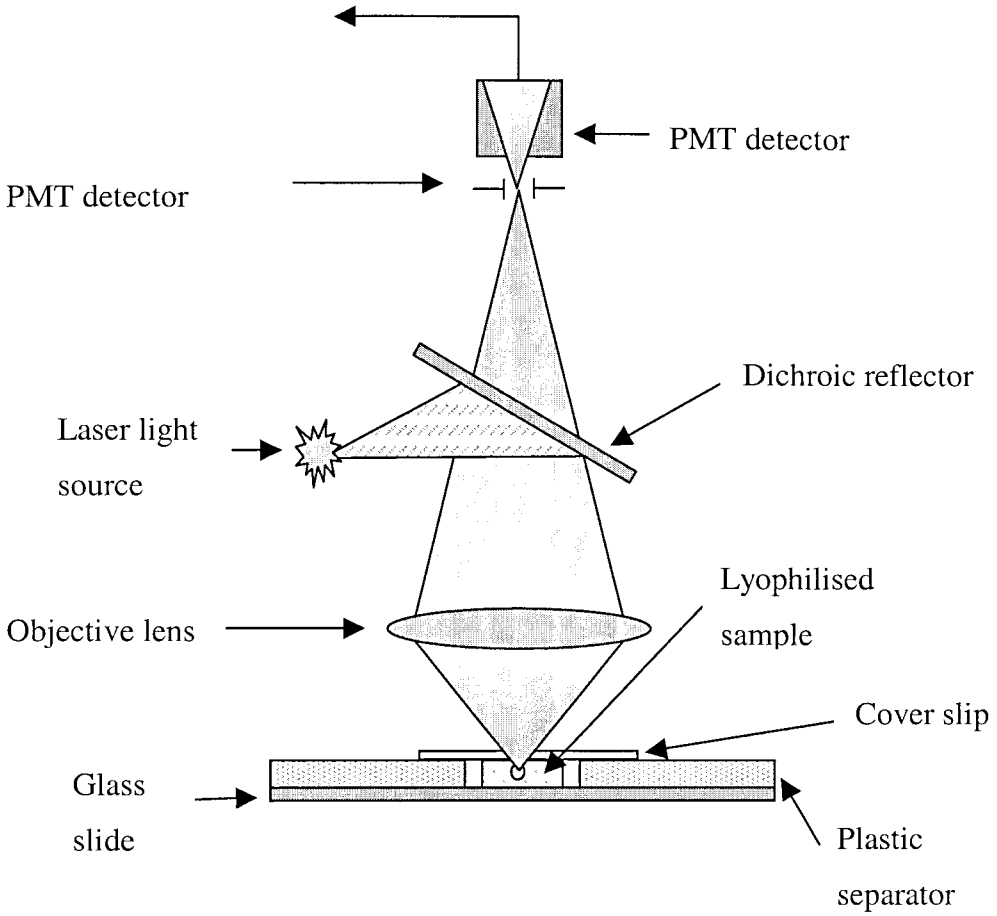


Figure 2.2 **Schematic representation of the Confocal laser scanning microscopy system.**

Re-drawn from the Bio Rad web site.

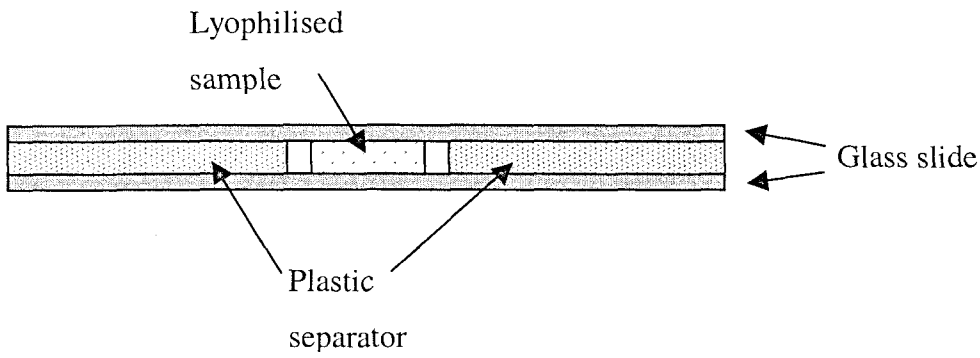


Figure 2.3 **Cross section of compression device for Confocal analysis samples.**

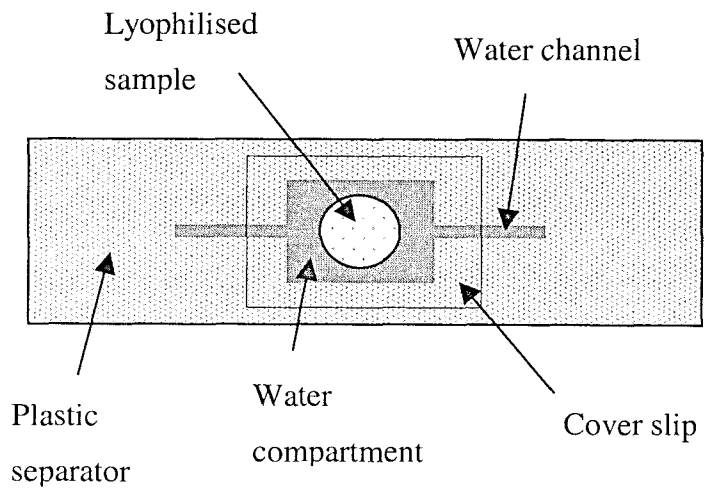


Figure 2.4 **Assembly prepared for Confocal Laser Scanning Microscopy.**

2.3 Results and Discussion

2.3.1 Effect of K4MP concentration

A typical profile obtained from the TA for adhesion of a lyophilised disc is shown in Figure 2.10. The peak force of detachment and area under the detachment force-time curve were measured for all formulations. The area under the force-time curve represents the actual work done by the TA to remove the formulation from the synthetic mucosal surface, whereas the peak detachment force is simply a measure of the maximum force recorded during that process.

Increasing the K4MP concentration of the formulations resulted in an increase in both the peak detachment force (Figure 2.11) and the AUC (Figure 2.12). The AUC appeared to increase in an exponential manner in response to K4MP concentration, suggesting that increasing the K4MP concentration increased the adhesivity of the formulation, and that the TA could detect this change in adhesion to the synthetic mucosal surface. Contact forces used were above those found in a previous study to represent a ceiling contact force (0.5N), above which no effect on measurements was observed (Wong et al., 1999).

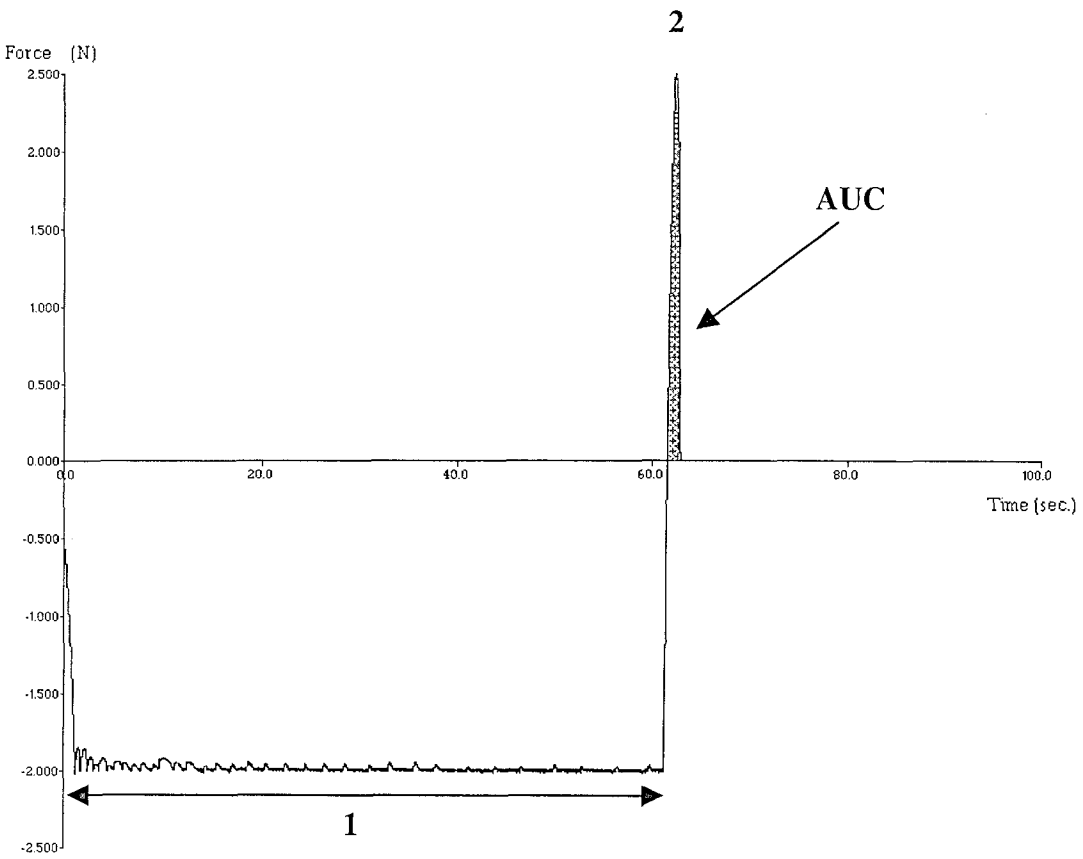


Figure 2.5 Typical TA adhesion profile.

1. Contact time and force
2. Peak detachment force

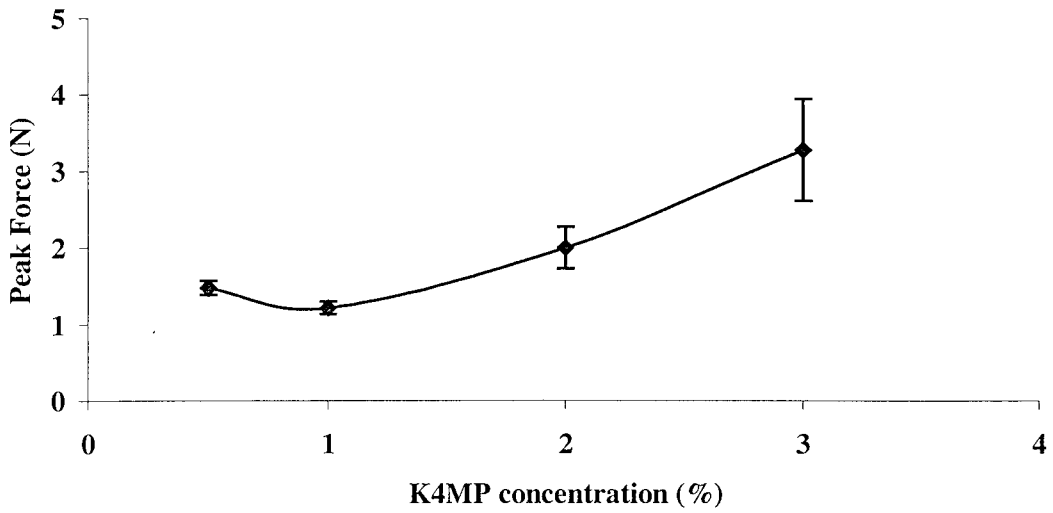


Figure 2.6 **Effect of K4MP concentration on peak detachment force (n=6).**

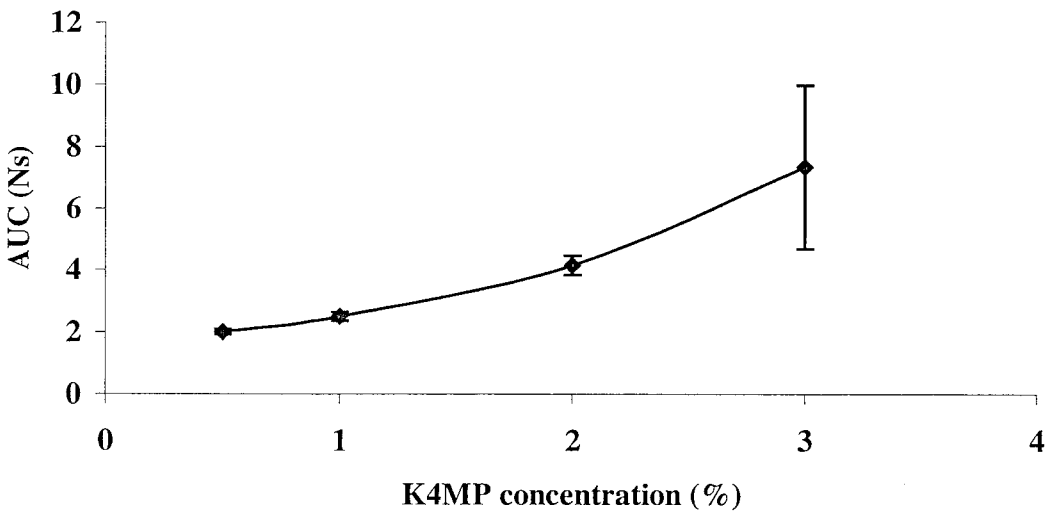


Figure 2.7 **Effect of K4MP concentration on AUC (n=6).**

2.3.2 Textural analysis of K4MP gels

The general trend for K4MP gels shows an increase in both peak detachment force and AUC as the polymer concentration increases, although some unexplained variation in this pattern is observed at concentrations of 0.75%-1% K4MP. The profiles for peak detachment force and AUC (figures 2.13 and 2.14 respectively) show that results obtained from the TA for the K4MP gels are significantly higher than the controls of agar alone, and agar with water.

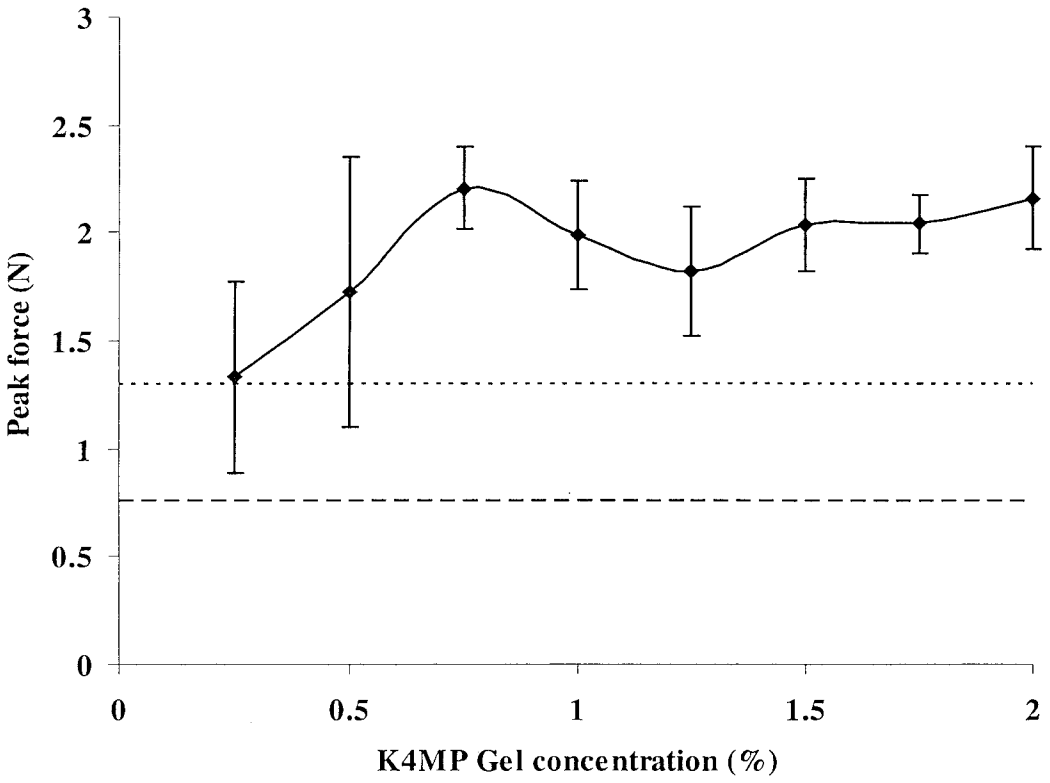


Figure 2.8 Effect of K4MP gel concentration on peak detachment force (n=6).

Controls of agar (dotted line) and water (broken line) are shown.

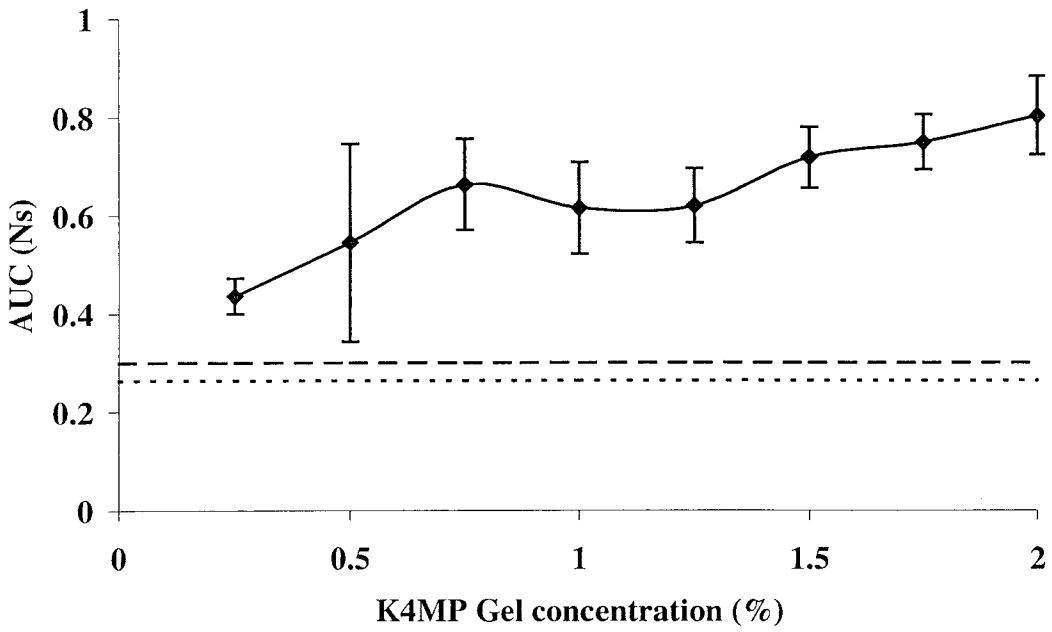


Figure 2.9 Effect of K4MP gel concentration on AUC (n=6).
Controls of agar (dotted line) and water (broken line) are shown.

When peak detachment force is considered at lower K4MP concentrations the gel formulation shows higher values than a lyophilisate of the same composition (Figure 2.15). However, examination of the AUC profiles, representing the total work done by the TA to remove the formulation from the synthetic mucosal surface, shows that lyophilisation results in a significant increase in adhesivity compared to its constituent gel (Figure 2.16). The increase in adhesion is expected, as the rehydrated lyophilisate would not absorb the same proportion of water to polymer as the original gel, which would effectively result in a gel of a much higher polymer concentration, offering greater adhesion. The measurement of peak force of detachment in the gels may represent only the cohesive break of the gel, rather than adhesion to the agar, explaining the discrepancy between these results and those for the AUC, a measurement of the total work done to overcome adhesive forces.

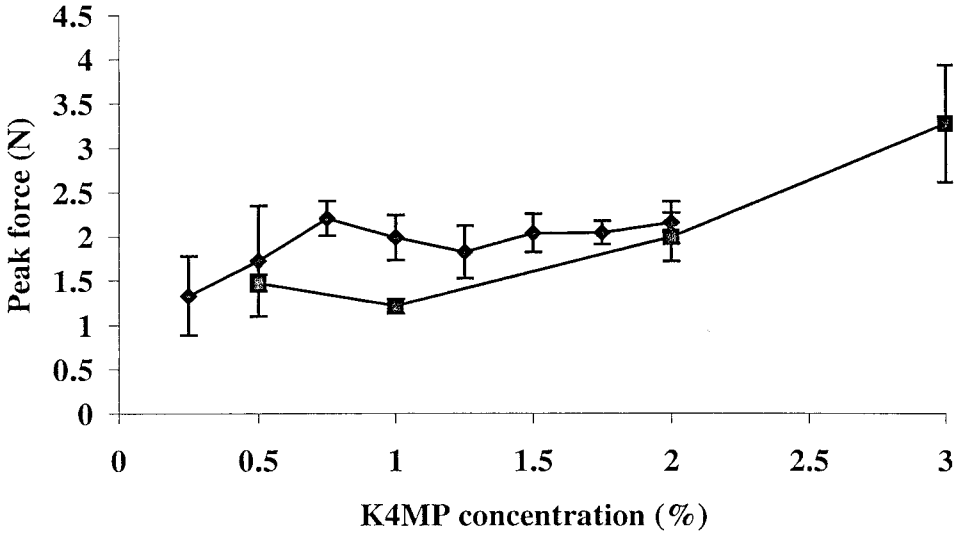


Figure 2.10 Comparison of peak force of detachment of K4MP gel and lyophilisate (n=6).

(◆) K4MP gel, (■) Lyophilised K4MP.

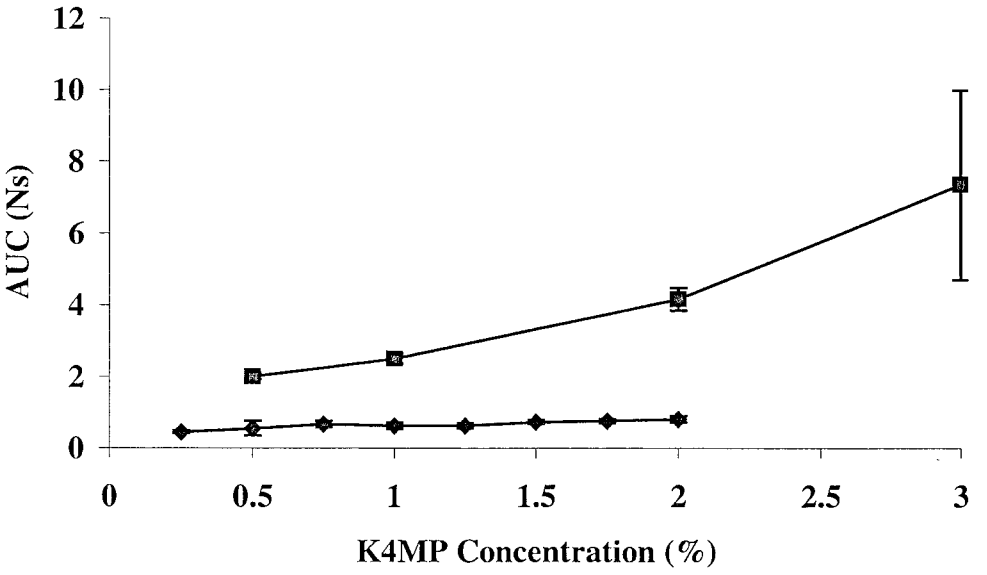


Figure 2.11 Comparison of AUC of K4MP gel and lyophilisate (n=6).

(◆) K4MP gel, (■) Lyophilised K4MP.

2.3.3 Influence of extent of hydration

Peak detachment force measurements from the TA were variable (Figure 2.17), and no differences could be detected between formulations of 1% or 2% K4MP or K100LV. Analysis of work of adhesion (Figure 2.18) produced greater reliability and reproducibility, and was able to show differences between the formulations. Initially, during the first 30 minutes of hydration, no differences could be detected. Further hydration of the lyophilisates showed that increasing molecular weight or concentration of polymer produced an increase in work of adhesion.

The extent of hydration had little effect on the work of adhesion of the K100LV formulations, but produced significant increases in adhesion of the K4MP lyophilisates, with the 2% K4MP lyophilisate showing superior adhesion to the 1% K4MP formulation. Overall adhesion increased in the order 1% K100LV < 2% K100LV < 1% K4MP < 2% K4MP. Results obtained for the TA infer that calculation of AUC for work of adhesion provides a more reliable measure of adhesivity. This is in agreement with a study carried out which found that tensile work provided a better means of assessing mucoadhesion than fracture strength of the gel (Hagerstrom and Edsman, 2001).

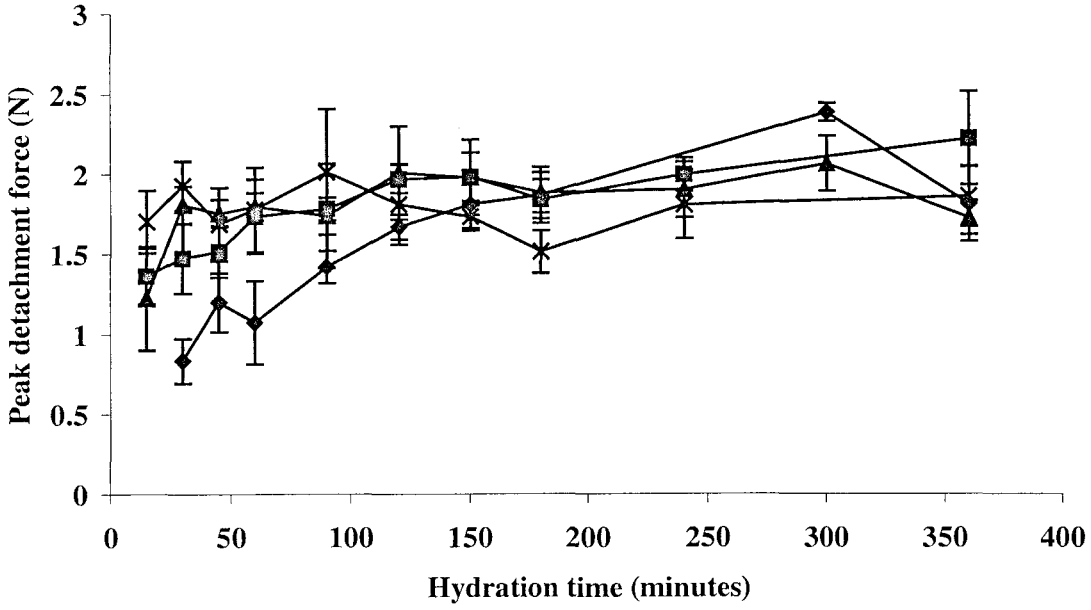


Figure 2.12 **Influence of extent of hydration and molecular weight on peak force of adhesion (n=6).**
(■) 1% K4MP, (◆) 2% K4MP, (x) 1% K100LV, (▲) 2% K100LV.

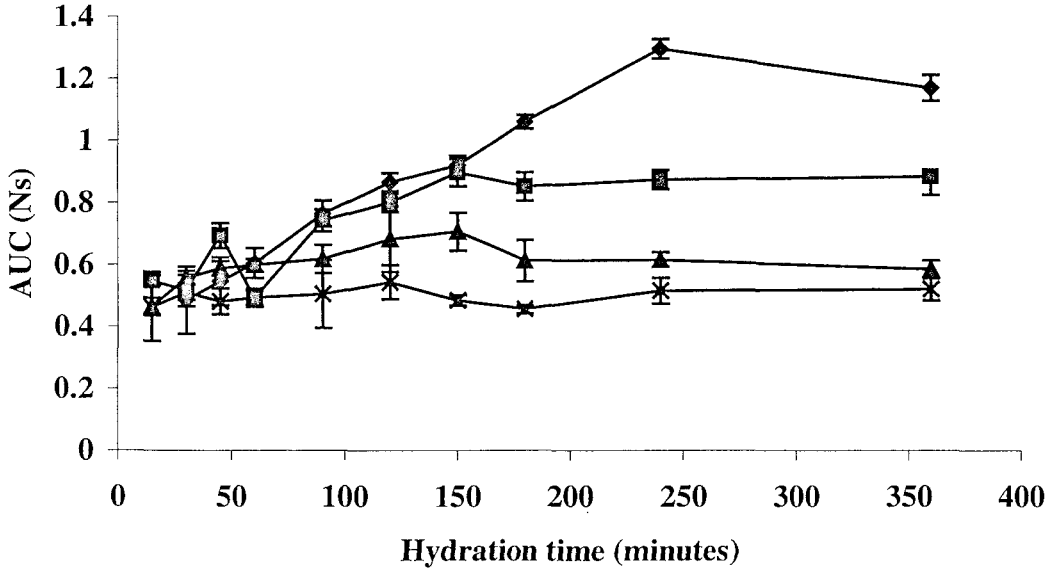


Figure 2.13 **Influence of extent of hydration and molecular weight AUC (n=6).**
(■) 1% K4MP, (◆) 2% K4MP, (x) 1% K100LV, (▲) 2% K100LV.

2.3.4 Simple hydration test

All formulations absorbed the equivalent of over ten times their weight in water during the course of the study period (Figure 2.19). Initial hydration was rapid, with rate of hydration in the first 60 minutes decreasing in the order 0.5%=1% > 2% > 3% K4MP. At 1 hour water uptake of the 0.5% K4MP formulation had begun to show a plateau effect, while 1, 2 and 3% formulations continued to hydrate at a slower rate, and were still undergoing hydration at the end of the study (360 minutes).

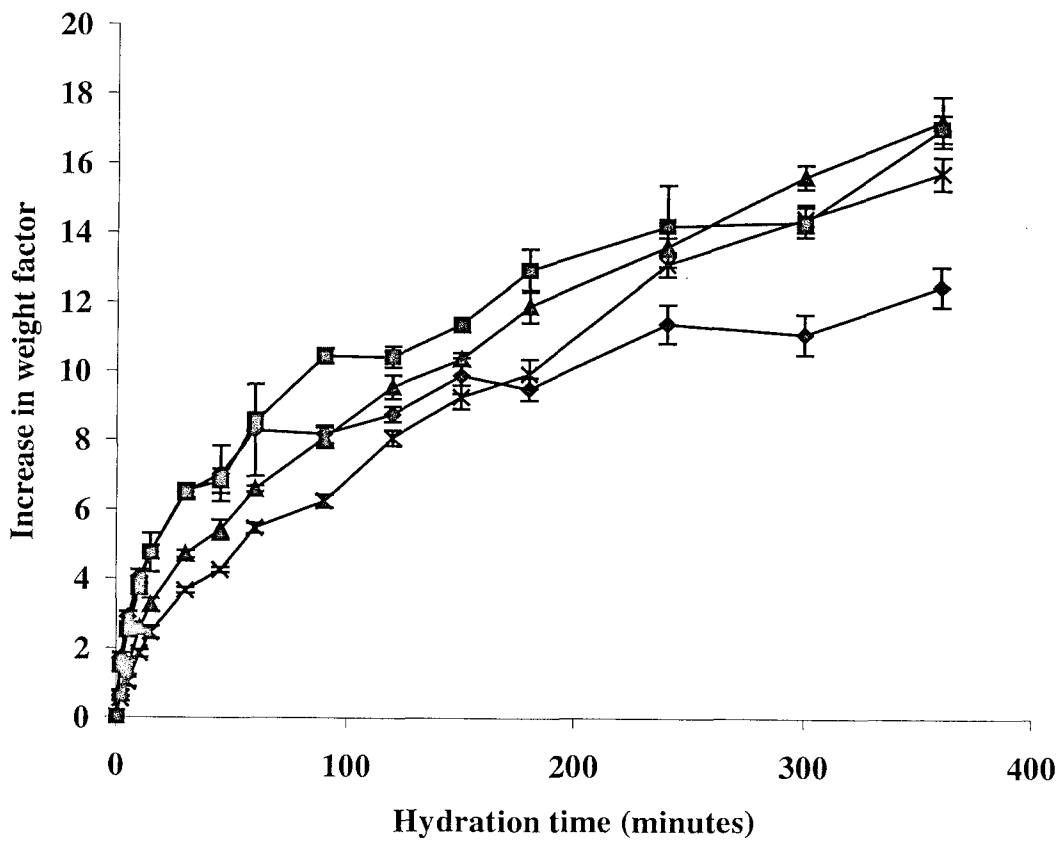


Figure 2.14 Hydration Profiles of the nasal insert formulations (n=6).
(◆) 0.5% K4MP, (■) 1% K4MP, (▲) 2% K4MP, (x) 3% K4MP.

Figure 2.20 shows images demonstrating differences in the extent of hydration and swelling in nasal inserts of differing HPMC concentrations. After 15 minutes the 0.5% K4MP formulation shows almost complete gel formation, 1% and 2% K4MP show partial gel formation, with 3% K4MP showing no gelled areas other than that in contact with the agar. At 240 minutes, all formulations have entirely hydrated to gel, although the spreading of the gel is greater as the K4MP concentration is reduced.

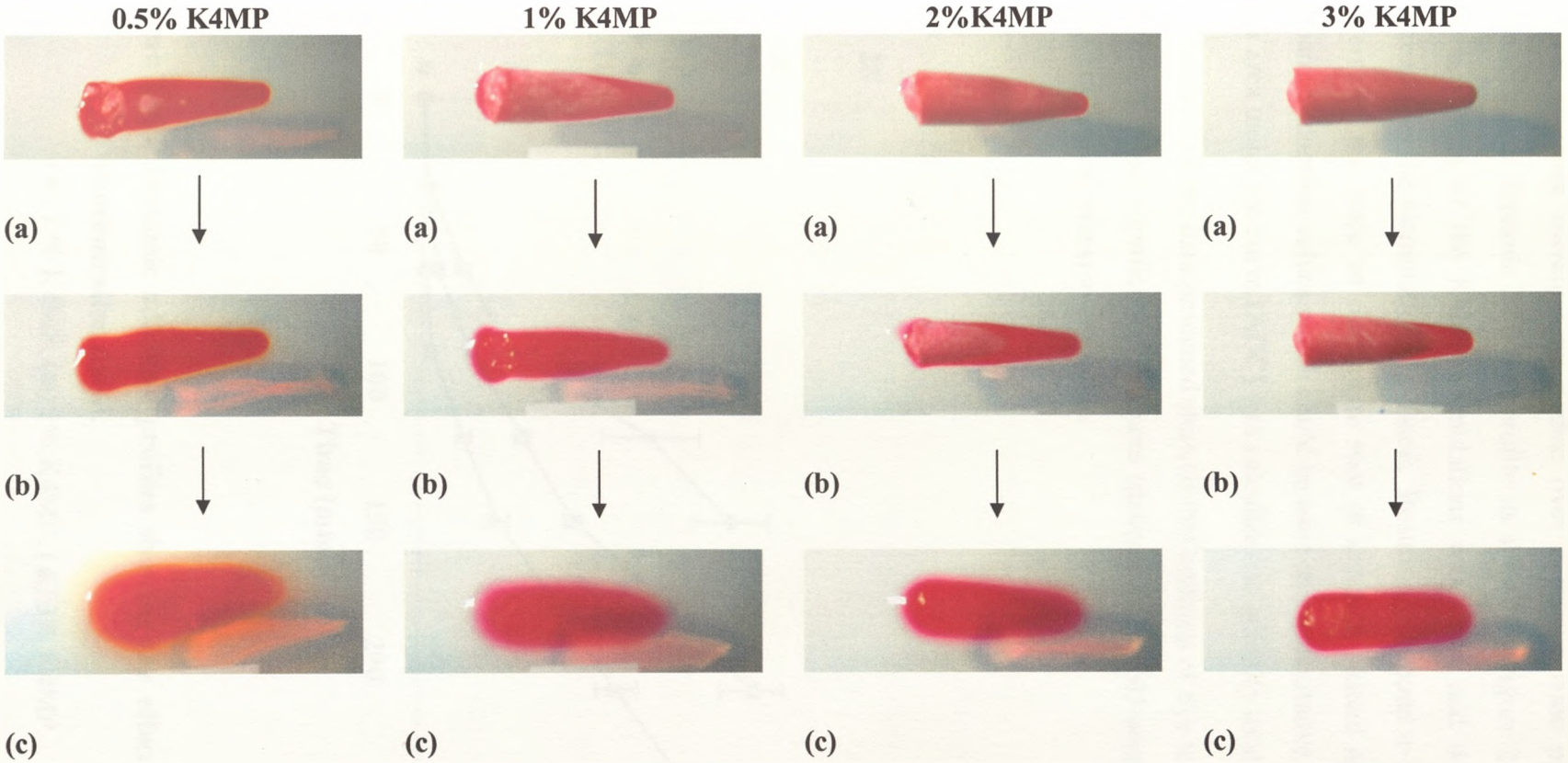


Figure 2.15 **Hydration and swelling of K4MP nasal inserts after (a) 15, (b) 60 and (c) 240 minutes on an agar synthetic mucosal surface.**

2.3.5 Dynamic adhesion test

The distance travelled by a disc over a known time period was measured, resulting in a dynamic adhesion profile as shown in Figure 2.21. During the test, initial sliding of the K4MP formulations was slow, and the rate of movement increased as the formulations hydrated. Results were found to be reproducible. The inverse of the slope of the square root of distance plotted against time produced dynamic adhesion values which could be used as a quantitative measure of adhesion. The area under the curve (AUC) was calculated to quantify total adhesion.

Analysis of the data produced showed that addition of dye to test formulations did not result in any significant differences (defined as $P > 0.05$ using a two-tailed, paired t-test) in kinetic adhesion constants.

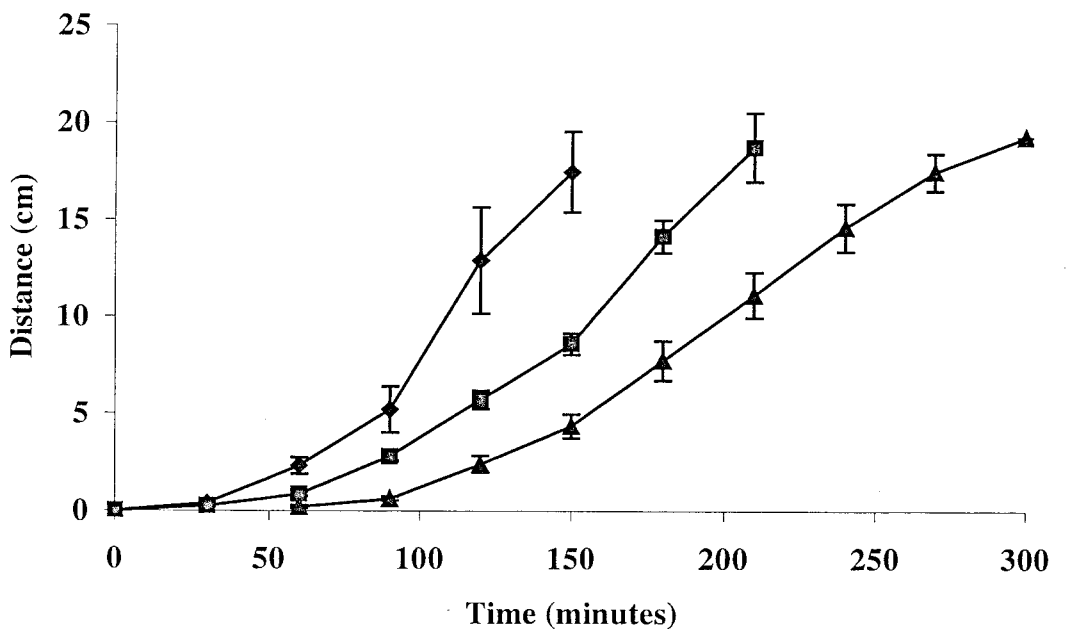


Figure 2.16 Dynamic adhesion profiles showing the effect of varying K4MP concentration (n=6).

(◆) 1 % K4MP, (■) 2% K4MP, (▲) 3% K4MP.

2.3.5.1 Effect of HPMC concentration and molecular weight

Lyophilised formulations which contained 1-3% K100LV and mannitol hydrated rapidly, and no results for a dynamic adhesion constant were obtained as the formulations had slid to the bottom of the 20cm test distance before the end of the first measurement interval of 30 minutes.

Data showing the effect of K4MP concentration and molecular weight on the dynamic adhesion value are shown in Table 2.4. Increasing K4MP concentration resulted in an increase in the dynamic adhesion value and AUC.

Table 2.4 Effect of HPMC concentration and molecular weight on dynamic adhesion value.

Formulation	Slope (± s.d.) (cm/min)	Dynamic adhesion value (min/cm)	AUC (cm.min)
1% K4MP	0.03063 (±0.0018)	32.65	878
2% K4MP	0.02189 (±0.0006)	45.69	1244
3% K4MP	0.01635 (±0.0011)	61.16	2035
1% K100LV	No Data	-	-
2% K100LV	No Data	-	-
3% K100LV	No Data	-	-

2.3.5.2 Effect of mannitol concentration

The effect of altering the mannitol content of the (2%) formulations on adhesion is shown in Table 2.5. Mannitol content did not significantly influence the adhesion behaviour of the K4MP lyophilisates.

Table 2.5 Effect of mannitol concentration on dynamic adhesion value.

Formulation	Slope (± s.d.) (cm/min)	Dynamic adhesion value (min/cm)	AUC (cm.min)
0.5% Mannitol	0.01900 (±0.0005)	52.63	1601
1% Mannitol	0.02189 (±0.0006)	45.69	1244
2% Mannitol	0.02030 (±0.0005)	49.25	1553
3% Mannitol	0.02048 (±0.0007)	48.83	1711

2.3.5.3 Effect of excipients

The effect of the addition of a number of excipients on the dynamic adhesion value is shown in Table 2.6. Substituting water soluble NaCl for mannitol in the formulation gave a dynamic adhesion value similar to lyophilisates containing mannitol. Replacement of mannitol with sucrose in a 2% K4MP lyophilisate resulted in an increase in dynamic adhesion values, and a slight increase in AUC. However, the hygroscopic nature of the sucrose resulted in the lyophilisates becoming sticky after a short time under ambient room humidity, and was therefore considered an unsuitable candidate for a practical formulation.

Incorporation of lactose in place of mannitol also resulted in an increase in dynamic adhesion value and AUC, although the lactose formulation did not display enough handleability to be considered a practical formulation.

Table 2.6 Effect of excipient concentration on dynamic adhesion value.

Excipient	Slope (±s.d.) (cm/min)	Dynamic adhesion value (min/cm)	AUC (cm.min)
3% NaCl	0.02035 (±0.0009)	49.15	1610
1% Sucrose	0.01627 (±0.0005)	61.46	1781
3% Sucrose	0.01805 (±0.0004)	55.41	1499
5% Sucrose	0.01816 (±0.0006)	55.06	1623
0.5% Lactose	0.01686 (±0.0004)	59.32	1783
1% Lactose	0.01587 (±0.0005)	63.01	1693
2% Lactose	0.01646 (±0.0004)	60.77	1797

The effect of addition of PVP polymer to the 2% K4MP/1% mannitol formulation is shown in Table 2.7. No formulation containing PVP displayed significantly greater adhesion than the 2% K4MP lyophilisate without PVP, and only the 1% and 2% PVP 10,000 lyophilisate produced similar adhesion, all other PVP formulations were significantly ($P<0.05$) less adhesive.

Increasing PVP concentration would generally be expected to increase adhesion (Jones et al., 2002), however findings show that, in general, increasing the PVP concentration results in a significant ($P<0.05$) decrease in the dynamic adhesion value and AUC, and that increasing PVP molecular weight also significantly decreased these parameters. These findings are similar to those of (Tobyn et al., 1996) who found that inclusion of PVP in mucoadhesive tablets significantly reduced adhesion (Tobyn et al., 1996). The authors suggested that as PVP polymers are hydrogen bond acceptors, they preferentially bond with hydrated polymers, resulting in a decrease in adhesion. This suggestion agrees well with the finding that increasing PVP content and molecular weight further decreased the dynamic adhesion value.

Table 2.7 Effect of PVP concentration and molecular weight on dynamic adhesion value.

Excipient	Slope (±s.d.) (cm/min)	Dynamic adhesion value (min/cm)	AUC (cm.min)
1% PVP 10,000	0.02164 (±0.0006)	46.20	1420
2% PVP 10,000	0.02152 (±0.0009)	45.84	1500
3% PVP 10,000	0.02442 (±0.0010)	40.94	1131
1% PVP 44,000	0.02496 (±0.0008)	40.06	1047
2% PVP 44,000	0.02512 (±0.0007)	39.81	1098
3% PVP 44,000	0.02968 (±0.0003)	33.69	789
1% PVP 70,000	0.02808 (±0.0006)	35.61	855
2% PVP 70,000	0.03453 (±0.0015)	28.96	778
3% PVP 70,000	0.03852 (±0.0038)	25.96	556

2.3.5.4 Effect of addition of active drug

Addition of NHT or bovine insulin to the formulation resulted in significant changes to the dynamic adhesion value. NHT and insulin formulations were both significantly more adhesive than the 2% K4MP formulation, with the NHT formulation giving a dynamic adhesion value of 50.45 and the insulin formulation a dynamic adhesion value of 67.04 compared with 45.69 for the formulation with no active drug. Corresponding AUC values for the NHT and insulin formulations were 1437 and 1877 respectively. The finding for insulin may be expected, as insulin might be expected to slow the rate of hydration of the formulation, resulting in a slower loss of adhesive properties. However, with such a theory, the presence of NHT in the formulation would be expected to decrease adhesion, as the water soluble NHT would encourage the absorption of water into the lyophilisate.

However, as shown in Table 2.5, changing the concentration of water soluble mannitol in the formulation did not alter adhesion values, therefore it may not be expected that the presence of another water soluble compound such as NHT would exert influence on hydration or adhesion. It may be concluded that in this case the presence of NHT in some way alters the nature of the formulation, in order to increase adhesion.

2.3.6 Texture analyser sliding adhesion

A typical TA profile obtained for the sliding adhesion test is shown in Figure 2.22. The peak force required to overcome adhesion was recorded as the peak initial force required to slide the formulation, and the area under the curve was calculated from the section 4 portion of the graph in Figure 2.22 as a measure of work done to overcome adhesion.

Initial adhesion of the formulations was high (Figures 2.23 and 2.24), although adhesion of the 3% K4MP formulation could not be recorded before 45 minutes, as it had not hydrated sufficiently to adhere to the agar synthetic mucosal surface. As the formulations became increasingly hydrated, adhesion was reduced and tended to plateau after approximately 2 hours.

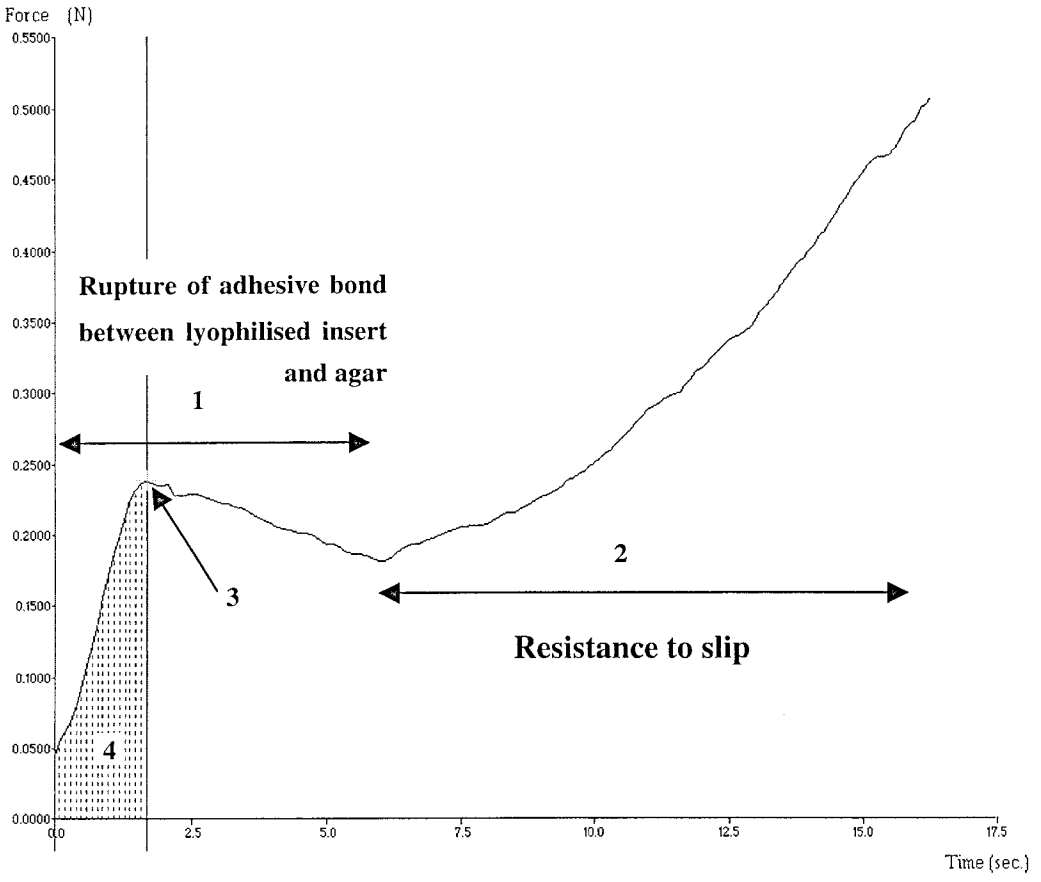


Figure 2.17 Typical TA sliding adhesion result obtained.

1. Force required to overcome adhesion of hydrated formulation to agar.
2. Subsequent sliding of semi-hydrated portion of formulation across surface of agar.
3. Peak Force.Area under the curve.

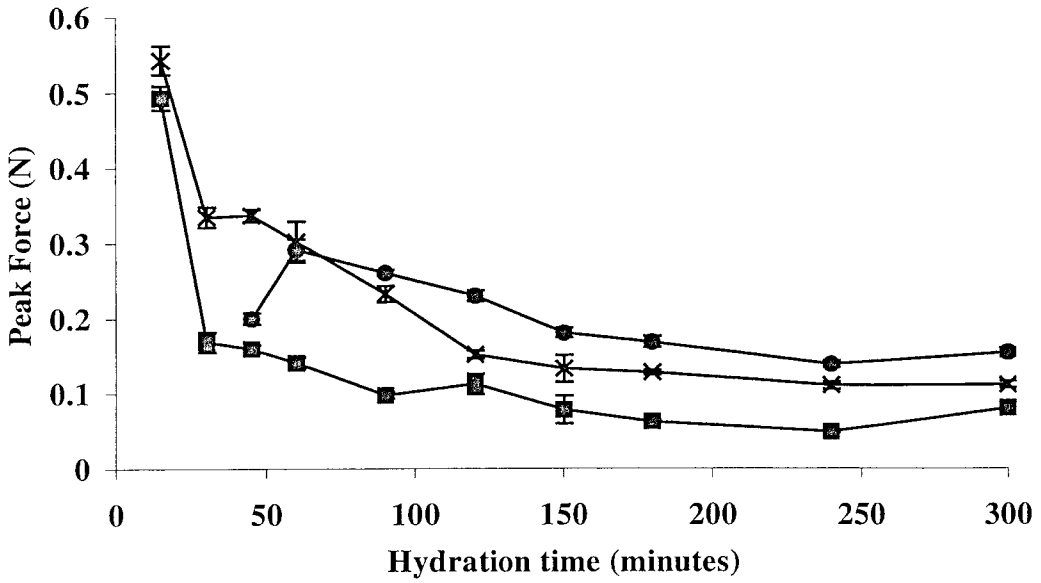


Figure 2.18 **Effect of extent of hydration on peak force of K4MP formulations in sliding adhesion test at a test speed of 2mm/s (n=6).**
(■) 1% K4MP, (x) 2% K4MP, (●) 3% K4MP.

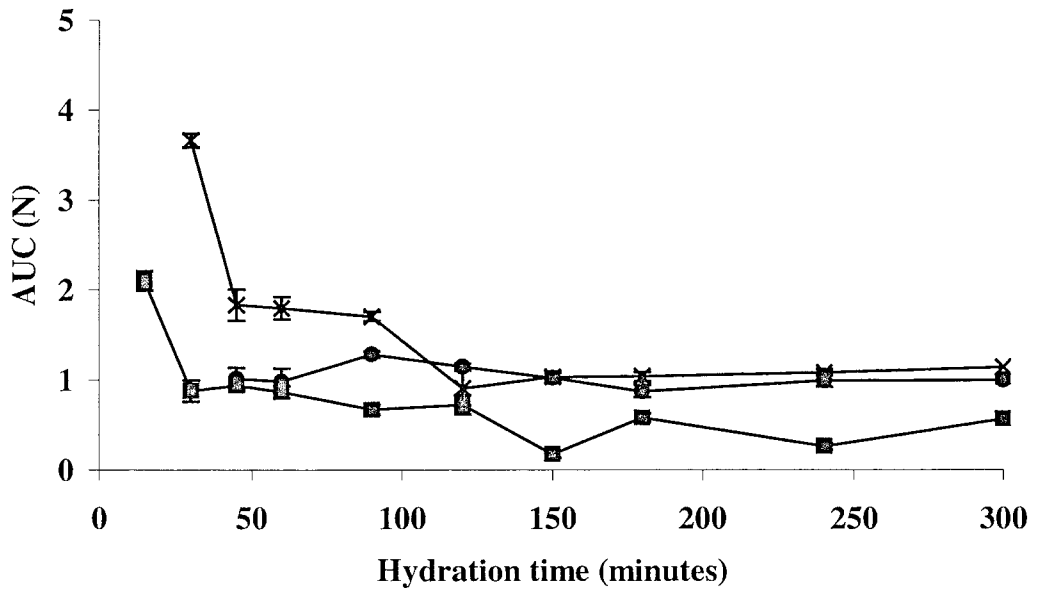


Figure 2.19 **Effect of extent of hydration on AUC of K4MP formulations in sliding adhesion test at a probe speed of 2mm/s.**
(■) 1% K4MP, (x) 2% K4MP, (●) 3% K4MP.

Overall, the 2% K4MP formulation was considered to display the optimal combination of adhesion in the sliding TA test, with high initial adhesion lasting approximately 90 minutes, before levelling out at an intermediate level of adhesion upon further hydration.

While the 3% K4MP lyophilisate displayed significantly higher adhesion in the latter stages of the test, the lack of initial adhesive effect would be a drawback *in-vivo* where only a short period of time would be available for a lyophilisate to establish initial adhesion. Decreasing the probe speed from 2mm/s to 1mm/s decreased the values obtained for AUC (Table 2.8), at the different stages of hydration.

The main drawback of the TA sliding adhesion test is that while it can quantify forces of adhesion after a specific period of hydration, it does not account for the spreading and movement of the gel in the nasal cavity by the cilia, an effect better represented in the dynamic adhesion test.

Table 2.8 Effect of changing K4MP concentration and probe speed at different stages of hydration.

Formulation	Initial AUC (Ns)		AUC at 240 minutes (Ns)	
	1mm/s	2mm/s	1mm/s	2mm/s
1% K4MP	4.87 ±0.23	0.88 ±0.12	0.90 ±0.02	0.26 ±0.04
2% K4MP	7.49 ±0.29	3.65 ±0.99	1.19 ±0.04	1.08 ±0.05
3% K4MP	1.52 ±0.06	1.01 ±0.12	1.36 ±0.06	0.99 ±0.06

2.3.7 Confocal laser scanning microscopy

Images captured from the Confocal Laser Scanning Microscope (CLSM) revealed that the rate of water ingress into the lyophilisates was dependant on:

a) The K4MP concentration of the lyophilisates

b) The section of the lyophilisate being studied

c) The addition of drug to the formulation.

The initial rate of water ingress at the edge of the lyophilisate was rapid compared to an apparent steady rate of water movement which was reached after a few minutes. For lyophilisates without drug, the rate of water ingress decreased in the order 0.5% K4MP > 1% K4MP > 2% K4MP > 3% K4MP, and CLSM images demonstrating the extent of initial hydration of the four polymer concentrations are shown in Figures 2.25 and 2.26. The fluroscein stained lyophilisate is green coloured in the image, while the rhodamine stained water is red. The rates of water ingress observed agree well with the findings of a study of swelling of hydrogels where increasing viscosity led to a decrease in water penetration (Michailova et al., 2000).

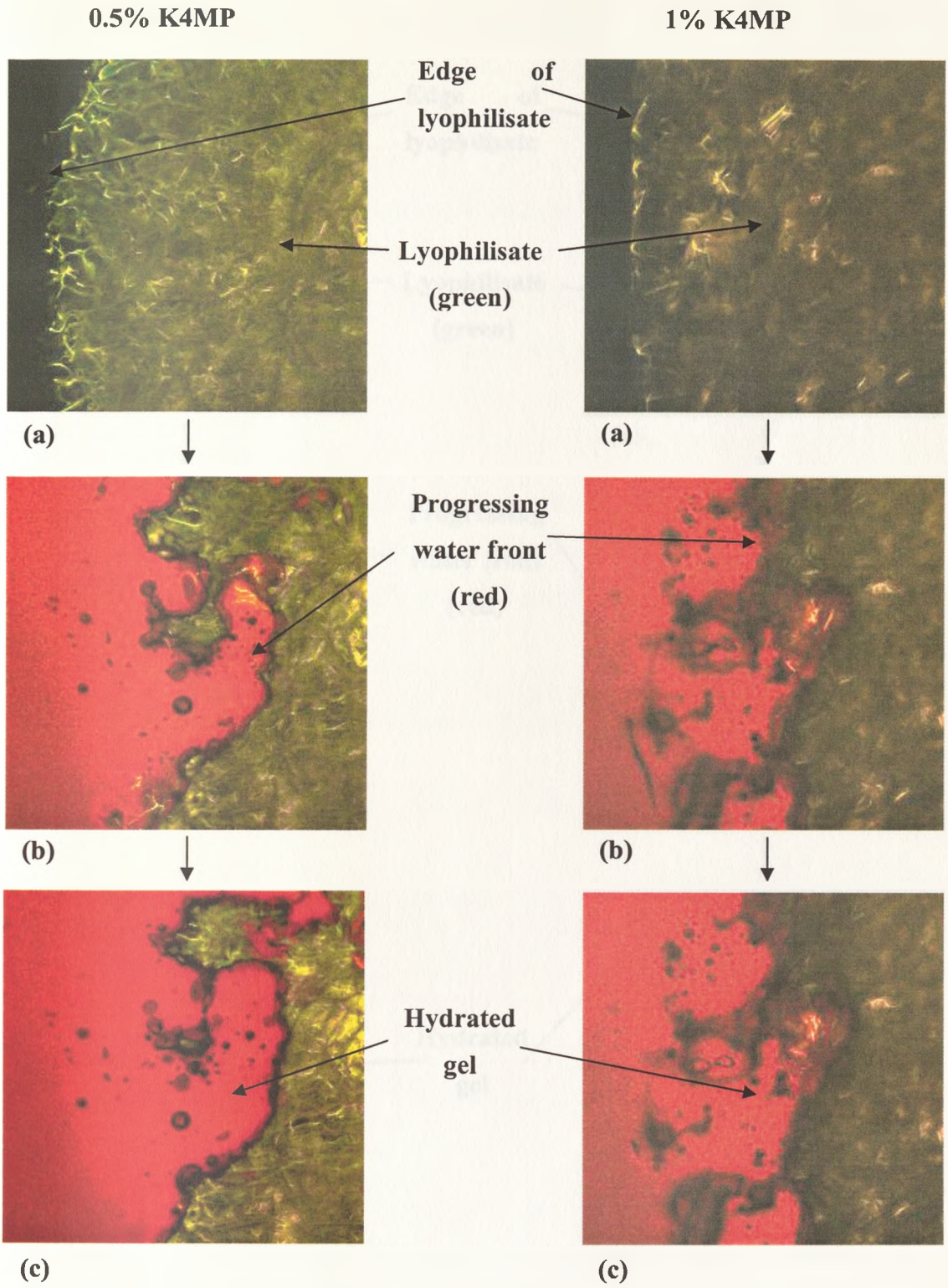


Figure 2.20 LSCM images of 0.5% and 1% K4MP lyophilisates at (a) 0 seconds, (b) 4 seconds and (c) 8 seconds following addition of water.

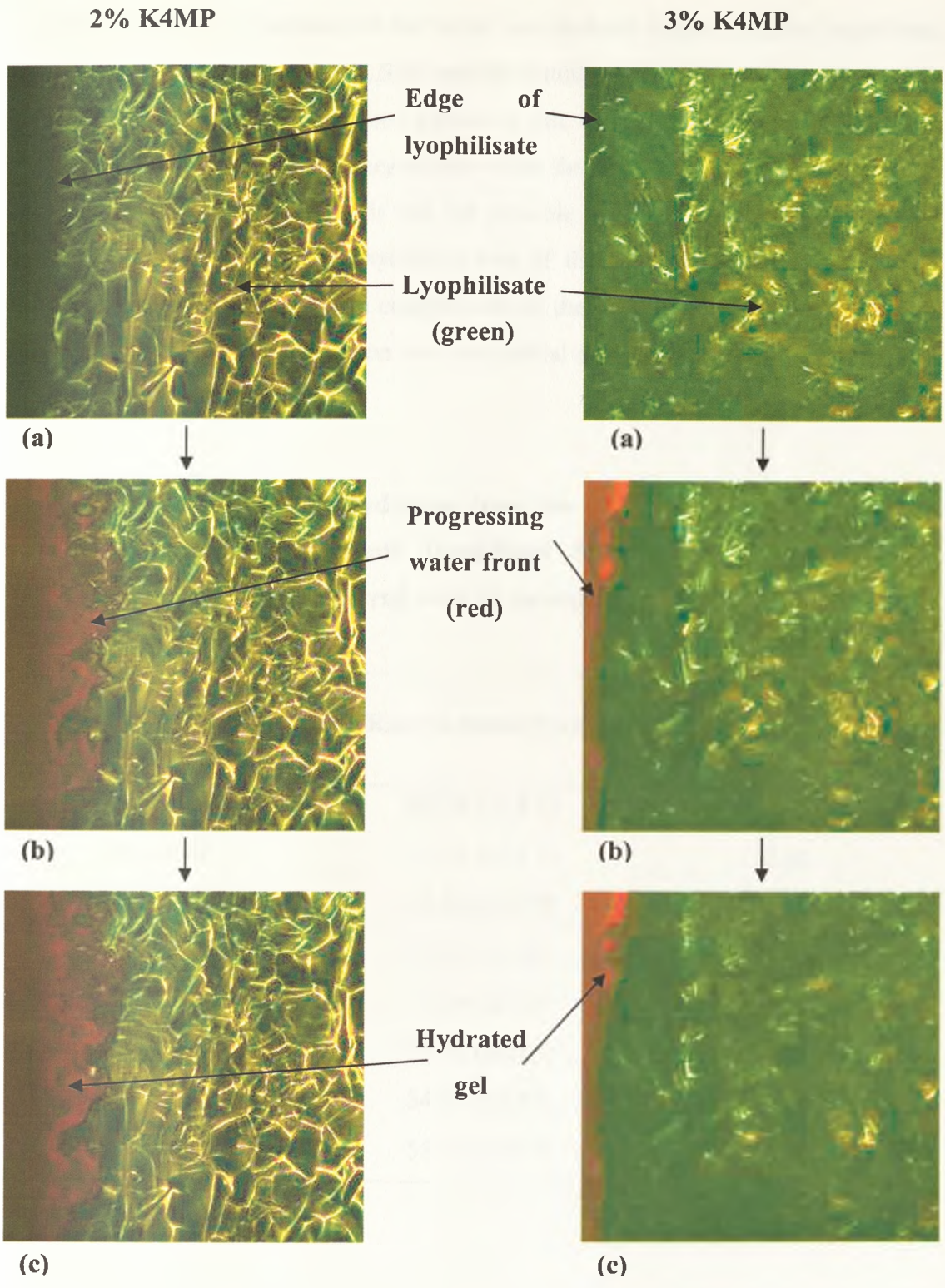


Figure 2.21 LSCM images of 2% and 3% K4MP lyophilisates at (a) 0 seconds, (b) 4 seconds and (c) 8 seconds following addition of water.

Table 2.9 shows a summary of the initial rate of water ingress into the lyophilised formulation studied using the CLSM, and the initial rate in comparison with the 2% K4MP formulation. The rapid and extensive rate of hydration of the 0.5% K4MP and 2% K100LV formulations meant that within the first minute the lyophilisate had completely formed a gel and so it was not possible to record a steady rate hydration value. A summary of steady hydration rate of the other formulations studied is shown in Table 2.10, along with comparisons to the 2% K4MP formulation and the extent of the reduction in hydration rate from initial measurements.

Table 2.9 Assessment of hydration from the CLSM showing initial rate of water ingress into lyophilised formulations (containing 1% mannitol), measured over 10 seconds.

Formulation	Rate in mm/s (± s.d.)	Compared to 2% K4MP (1% mannitol)
0.5% K4MP	80.58 (±18.2)	3.33
1% K4MP	52.25 (±11.7)	2.16
2% K4MP	24.20 (±3.70)	1.00
3% K4MP	9.21 (±5.40)	0.38
2% K4MP (no mannitol)	13.34 (±3.2)	0.55
2% K4MP/2mg NHT	31.90 (±10.3)	1.32
2% K4MP/49iu insulin	34.52 (±5.62)	1.43
2% K100LV	55.13 (±14.2)	2.28

Table 2.10 **Assessment of hydration from the CLSM showing steady rate of water ingress into lyophilised formulations, measured over 3 minutes.**

Formulation	Rate in mm/s (± s.d.)	Compared to 2% K4MP	Percentage of initial rate
0.5% K4MP	-	-	-
1% K4MP	3.46 (±0.53)	3.53	6.62
2% K4MP	0.98 (±0.07)	1.00	4.05
3% K4MP	0.41 (±0.11)	0.42	4.45
2% K4MP (no mannitol)	0.70 (±0.04)	0.71	1.00
2% K4MP/2mg NHT	0.52 (±0.15)	0.53	1.63
2% K4MP/49iu insulin	0.70 (±0.14)	0.71	2.03
2% K100LV	-	-	-

Reduction of initial hydration rate to the steady rate was of a similar magnitude for K4MP lyophilisates with no active drug content, steady rate being 4-7% that of initial measurements. Steady state hydration rate was further slowed in lyophilisates containing NHT and insulin, with these formulations displaying a reduction to 1.5-2% that of initial rates. Removal of mannitol from the 2% K4MP formulation resulted in a decrease in the rate of hydration.

During the course of the CLSM study, an instantaneous "flash" effect was occasionally observed on addition of water which was hypothesised to be a result of the mannitol in the formulation being rapidly dissolved and providing an osmotic pathway to conduct a very small amount of water through the polymer matrix, while not being of sufficient quantity to result in gel formation. This theory may be compatible with the finding that removal of mannitol from the 2% K4MP formulation resulted in a decrease in the rate of hydration. The presence of highly water soluble mannitol in the formulation may aid the ingress of water into the

formulation by providing such a pathway, the "flash" absorption of a small volume of water pre-hydrating the polymer, meaning that slightly less water need be absorbed to hydrate the polymer, therefore increasing the rate at which hydration occurs.

Addition of 2mg of water soluble NHT further increased the rate of water ingress into the 2% K4MP lyophilisate (with mannitol), again suggesting that the presence of water soluble compounds aid the hydration of the formulations. However, addition of insulin to the 2% K4MP (with mannitol) formulation resulted in a slight further increase in rate of hydration, an unexpected finding, as the presence of a large molecule such as insulin might be expected to impede the ingress of water into the system. This may explain the results from the Dynamic Adhesion test, where the insulin formulation was found to be more adhesive than the NHT formulation, perhaps as a result of the insulin lyophilisate hydrating more rapidly to form an adhesive gel.

Formulation parameters which increase water ingress into the lyophilisates are likely to provide a more rapid adhesive effect through formation of the polymer gel. In particular, the initial water ingress is of importance (Hedenus et al., 2000), as it is this surface which will provide an interface with the mucosal surface and be involved in forming initial adhesive bonds. Conversely, if the initial rate of hydration is too high, over-hydration may occur, resulting in a lack of useful adhesive properties (Mortazavi, 1995). The order of rate of initial hydration in the CLSM confirms that found in the simple hydration test, and also confirms the reduction in hydration rate observed. The lack of adhesion demonstrated by K100LV formulations in the dynamic adhesion test demonstrates the effect of over-hydration of the formulations on adhesive capabilities.

The apparent decrease in adhesion on the sliding TA test after 90 minutes further demonstrates the effect of over-hydration, coupled with the gradual decrease in adhesion during the dynamic test, after approximately 90 minutes. This effect is similar to that described in a study which found that increasing water content of Carbopol 934P gels decreased adhesiveness and cohesion (Mortazavi and Smart, 1993). In that study it was concluded that water uptake might be of more relevance to adhesion than surface molecular interactions. A further study concluded that an

increased rate of swelling resulted in a decreased duration of adhesion (Mortazavi and Smart, 1994), as observed here with the TA. The importance of the initial rate of hydration manifests in results from the sliding TA test, where the 3% K4MP formulation displayed a lack of initial adhesion due to lack of hydration. The reduction of adhesion of lyophilisates on addition of PVP in the dynamic adhesion test further demonstrated the importance of hydration in adhesive properties.

2.4 Conclusion

Hydration kinetics of lyophilised formulations have a direct influence on adhesivity, and are therefore of paramount importance when designing bioadhesive formulations. Ideally a combination of hydration tests as described in this chapter would be used to assess the properties of prospective formulations to obtain an overall picture of the hydration/adhesion process, removing individual limitations of a single method. Many researchers have reported similar thoughts, presenting a collection of different techniques to create an overall picture of the processes taking place (Hagerstrom and Edsman, 2001, Mortazavi and Smart, 1994, Jones et al., 1997, Eouani et al., 2001).

HPMC polymer molecular weight and concentration also contribute greatly to the adhesion of the lyophilisates, with increase in each generally providing an increase in adhesion. However, the sliding adhesion test suggests that too high a polymer concentration may be detrimental to adhesive effect, most likely due to the resulting impedance of the compact polymer matrix on water ingress and hydration, as observed in the CLSM and simple hydration test. The lack of expression of this effect in the dynamic adhesion test further demonstrates the necessity of using a combination of tests in evaluating adhesion.

An ideal bioadhesive formulation is most likely to be that which displays an optimal combination of properties in the above test, rapid initial hydration and therefore adhesion, without becoming over-hydrated too quickly resulting in loss of adhesion.

Chapter 3 Physical Properties of Lyophilised Dosage Formulation

3.1 Introduction

Examination of the physical properties of a dosage formulation is important in order to evaluate storage requirements, and attempt to assess the behaviour of the dose *in-vivo*. Many release studies concerning polymers rely on the USP or BP rotating basket method (Cheong et al., 1992, Mitchell et al., 1993, Miyazaki et al., 1995) or its adaptations, and also the USP dissolution apparatus (Hosny, 1993, Ahn et al., 1995). One problem with the USP dissolution apparatus is that bioadhesive formulations will have a tendency to randomly stick to different parts of the dissolution apparatus, leading to variability of results. Attempts to overcome this problem have included using two layers of mesh to trap the dosage form under the paddle, as reported by (Durig and Fassihi, 2000). Other similar methods have been reported (Illum et al., 1987, Aikawa et al., 1998, Lemoine et al., 1998), with the formulation directly in contact with the liquid into which the drug is to diffuse. However in such systems the formulation being studied is immersed in water, and this does not particularly represent the conditions in the nasal cavity.

Therefore alternative systems have been developed in an attempt to mimic the conditions in the nose. This generally involves the use of a filter or membrane of some sort at an air:liquid interface on which the formulation can be placed. The release of the drug through the membrane can then be studied by extraction of liquid from the receptor compartment of the set up (Witschi and Mrsny, 1999). In some cases the donor side is enclosed in order to mimic the humid conditions in the nasal cavity (Cornaz et al., 1996).

Dynamic Vapour Sorption (DVS) has recently become available as a useful tool for the rapid assessment of vapour sorption properties of materials. To date, the published literature concerning the use of the DVS equipment has generally focused

on its role in determining the extent of amorphous and crystalline content in powders such as lactose (Buckton and Darcy, 1995, Lane and Buckton, 2000, Buckton and Darcy, 1999). Researchers have demonstrated that in mixed powders of amorphous and crystalline lactose, crystallisation of the amorphous regions was observed as the relative humidity (RH) was increased to ~50% (Buckton and Darcy, 1995). This crystallisation event manifested itself in the DVS as a decrease in weight (percentage weight of the dry weight of the powder), following a steady weight increase due to sorption of water up until that point.

As lactose is known to form a monohydrate upon crystallisation, the authors were able to calculate the theoretical overall weight increase of a known composition amorphous:crystalline lactose powder. The weight increase would be due to water being held in the newly formed monohydrate after one sorption/desorption cycle. The theoretical values were then compared with those obtained experimentally, and a good correlation was found between the known amorphous content of the powder, and the experimentally calculated content. Further data published (Buckton and Darcy, 1999) however, suggest that as higher proportions of amorphous lactose are included in the powder mixture, not all of the sample will crystallise to form the monohydrate. This was demonstrated by experimentally calculated values which were increasingly falling below actual amorphous content.

The reason for crystallisation of amorphous material at a particular RH is related to the glass transition temperature (Tg) of the substance. The Tg is the temperature above which high molecular mobility exists, and below which "the material changes on cooling from a liquid or rubbery state to a brittle state" (Craig et al., 1999). Sorption of water vapour results in the lowering of the Tg to below room temperature, as the water acts as a plasticiser, increasing molecular mobility and allowing crystallisation to occur. A decrease in weight is then observed, as water which had been contained within amorphous regions is expelled on crystallisation. This process will also occur at temperatures below Tg, but may be extremely slow (Craig et al., 1999).

Other analysis techniques available for the study of amorphous materials include solution calorimetry (Hogan and Buckton, 2000), modulated temperature differential

scanning calorimetry (MTDSC) (Saklatvala et al., 1999, Craig et al., 2000) and micro-thermal analysis (Craig et al., 2002).

The occurrence of recrystallisation is of relevance to storage of materials. If slow water desorption existed in such a collapsed material, the sample might show no "loss on drying", and be thought to be completely dry (Buckton and Darcy, 1999). However, the collapsed amorphous material will eventually crystallise, irrespective of the Tg (as previously mentioned), and water previously contained within the amorphous regions will be released, making the sample "noticeably wet". This will have obvious implications for the stability of the sample. Also, such samples will require storage under conditions which do not expose it to threshold levels of humidity.

3.2 Materials and Methods

3.2.1 Chemicals

HPMC powder (K4MP and K100LV grades) was obtained as a gift from Dow Chemicals (Michigan, USA) and D (-) mannitol powder (GPR) was purchased from BDH (VRW, Poole, UK). Nicotine hydrogen tartrate (NHT), Insulin (human recombinant expressed in Ecoli and bovine) and Phosphate Buffered Saline (PBS) pH 7.4, were purchased from Sigma (Gillingham, UK). Bio-Rad Protein Assay was purchased from Bio-Rad Ltd. (Hemel Hempstead, UK).

3.2.2 Apparatus

Polythene microcentrifuge tubes (0.2mL) were obtained from Life Sciences, UK. Whatman Filter Paper (Grade 1, 42.5mm diameter) was purchased from BDH (VRW, Poole, UK). Small volume (700μL) far UV quartz spectrophotometer microcells of path length 1cm were purchased from BDH (VRW, Poole, UK). A UV double beam spectrophotometer (Helios Alpha or UV1, Thermospectronic, Rochester, USA.) was used in the analysis of insulin content of samples using the BioRad Protein Assay. A Virtis Advantage Freeze Drier, which carried out a cycle with preset stages, was used to lyophilise HPMC gels (BioPharma Process Systems, UK). A Polaron SC515 SEM Coating System (Bio-Rad sputter coater, Bio-Rad Ltd., Hemel Hempstead, UK) was used to coat samples for SEM analysis, and. SEM images were obtained using a Phillips SEM 500.

3.2.3 Methods

3.2.3.1 Preparation of HPMC gels

HPMC gels were prepared as described in section 2.2.3.1

HPMC gels containing NHT were prepared as described in section 2.2.3.1.

HPMC gels containing insulin were prepared as described in section 2.2.3.1.

3.2.3.2 Preparation of lyophilised formulations

HPMC gels were lyophilised as described in section 2.2.3.2.

3.2.3.3 Distribution of insulin in lyophilised nasal inserts

In order to confirm that the lyophilised formulations contained an even distribution of the insulin added to the preparation, a sample of each formulation studied was analysed for insulin content. Each sample was divided into three approximately equal sections (top, middle and bottom), which were weighed, and then allowed to dissolve in 10mL PBS for two hours. Appropriate dilutions were carried out in order to bring the insulin concentration of the solution into the 1-20μg/mL range of the insulin assay. The insulin content of each of the samples was then assessed using a Bio-Rad protein assay which is based on the binding of the agent Coomassie Brilliant Blue G-250 to protein, and undergoing a colour change from red to blue. This results in a change of the absorption maximum of the dye from 465 to 595 nm, allowing the quantity of protein present to be assessed using visible range spectrophotometry (Bradford, 1976). The assay was performed by vortexing 0.2mL of the dye reagent with 0.8mL of the insulin containing sample, allowing the colour change reaction to take place, and measuring the absorbance at 595 nm. Insulin content was calculated from a standard curve previously prepared.

3.2.3.4 *In-vitro* release of insulin from lyophilised nasal inserts

The *in-vitro* release of insulin from lyophilised nasal inserts was studied using a diffusion chamber based on a design previously described by Cornaz et al., 1996, intended to mimic conditions within the nasal cavity. The diffusion chamber consisted of a receptor and donor compartment, separated by a filter paper membrane which is maintained just in contact with the surface of the dissolution media contained within the receptor compartment. This means that the filter paper is sufficiently wetted to allow hydration of the formulation, without submerging the test dosage in dissolution media. The donor compartment was sealed after the formulation was placed on the filter paper membrane, to ensure that it contained air saturated with water. The individual components of the dissolution apparatus are shown in Figure 3.1, and the complete dissolution assembly is shown in Figure 3.2.

The receptor compartment, which contained PBS (pH 7.4) was constantly agitated with a magnetic stirrer, and the dissolution chamber was maintained at 37°C in a water jacket. The system was left to equilibrate for one hour before use. The test formulation was placed on the filter paper and 0.8mL samples were removed from the dissolution media for analysis of insulin content using the Bio-Rad protein assay as described in section 3.2.3.3. Samples removed were replaced with an equal volume of fresh PBS.

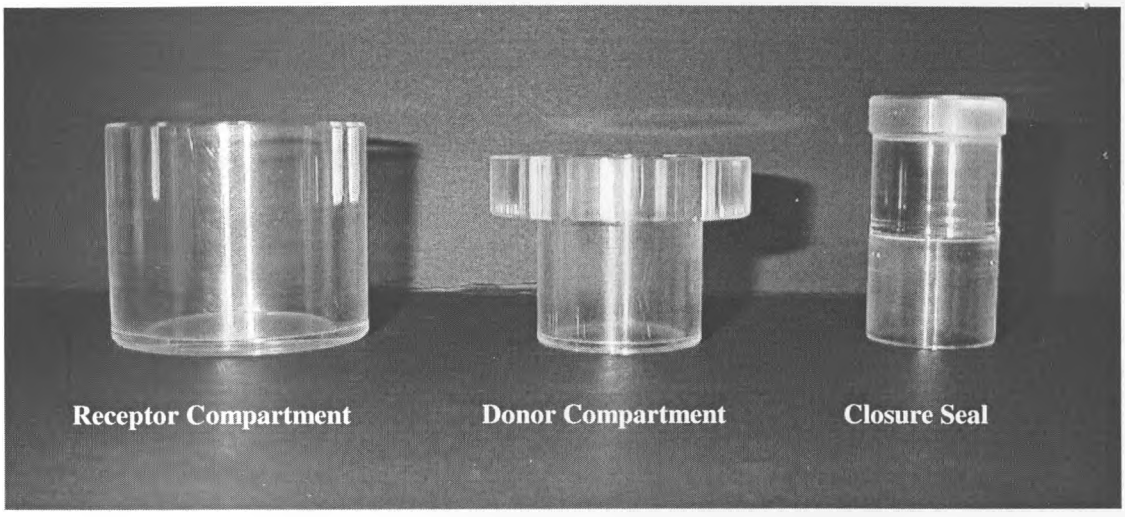


Figure 3.1 Individual components of the dissolution apparatus.

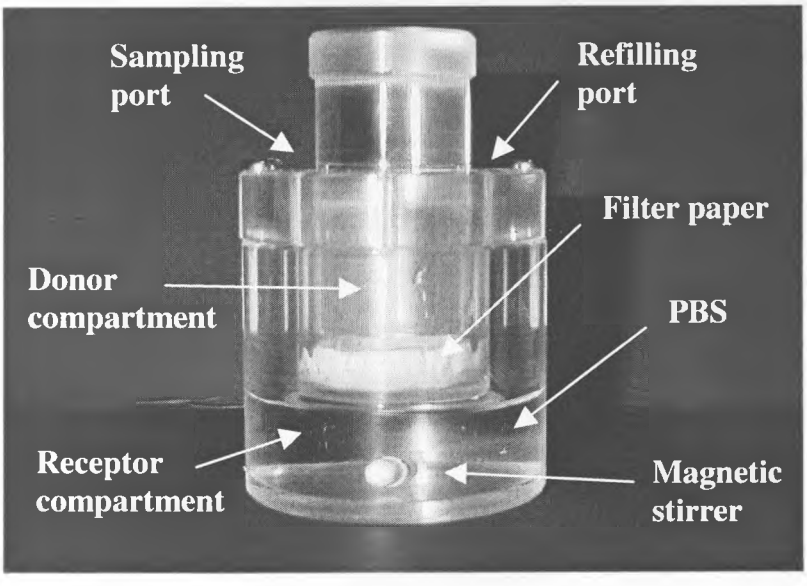


Figure 3.2 Assembled dissolution apparatus.

3.2.3.5 Scanning electron microscopy

Lyophilised samples were prepared by removing a section of the lyophilisate with a sharp scalpel blade, in such a way as to expose an internal area without destroying the structure, fixing the sample in place by means of copper electrical tape and gold coating the sample using a Polaron SC515 SEM Coating System (Bio-Rad sputter coater, Bio-Rad Ltd., Hemel Hempstead, UK). Powder samples were sprinkled onto the copper tape and coated in the same manner. SEM images were obtained using a Phillips SEM 500, with a spot size of 320Å and 12KV intensity. The average pore size of the internal structure of lyophilised inserts was determined by measuring a random sample of the pores (n=10) on the SEM images, and comparing with a known measurement scale.

3.2.3.6 Dynamic vapour sorption

The Dynamic Vapour Sorption apparatus (Figure 3.3) consists of a highly sensitive Cahn microbalance, and a chamber within which the humidity and temperature can be regulated as required via a computer system (Figure 3.4), allowing any weight changes in a sample due to sorption or desorption of water vapour to be accurately measured. Analysis of the data produced can provide information on the stability and general behaviour of the product at particular humidities, and any chemical or physical changes which may occur as a result of the sorption or desorption of water. To date, the published literature concerning the use of the DVS equipment has generally focused on its role in determining the extent of amorphous and crystalline content in powders such as lactose.

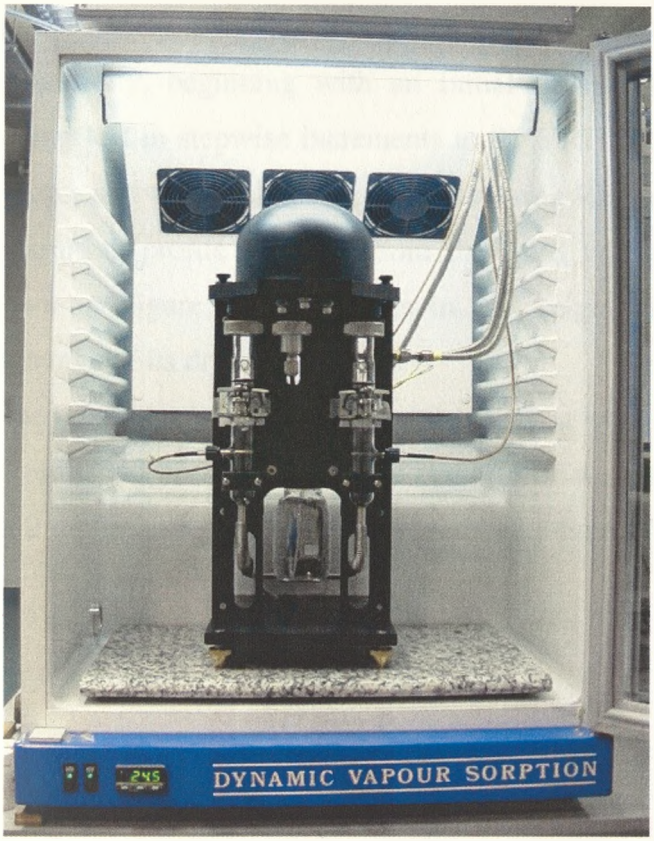


Figure 3.3 **The Dynamic Vapour Sorption apparatus.**

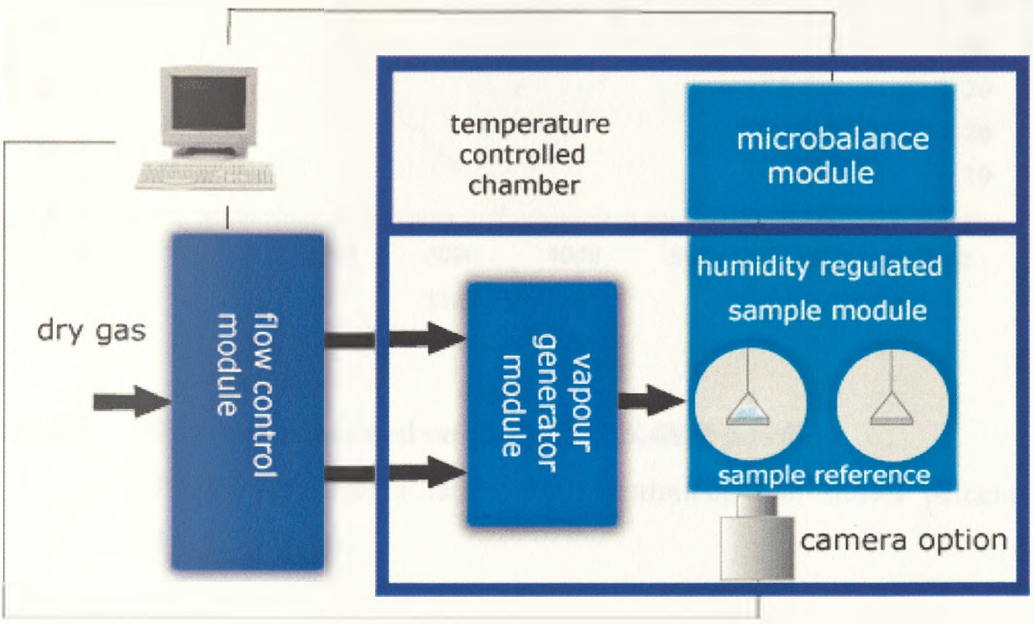


Figure 3.4 **Schematic representation of the Dynamic Vapour Sorption system**
Reproduced from Surface Measurement Systems website.

Samples of approximately 10-20mg were subjected to a controlled cycle of changing relative humidity, beginning with an initial drying phase at 0% RH, followed by increasing RH in stepwise increments to 95% RH. RH was decreased through the same steps, and these increasing and decreasing RH cycles repeated. A typical sorption/desorption profile obtained from the DVS with the stepwise RH programme is shown in Figure 3.5. Changes in the weight of the sample are expressed as a percentage of its dry weight.

Progression to the next increment of a cycle was triggered when either the weight of the sample had remained constant, such that the rate of weight change over 20 minutes was < 0.002 mg/minute, or that the maximum step time of 999 minutes had been reached.

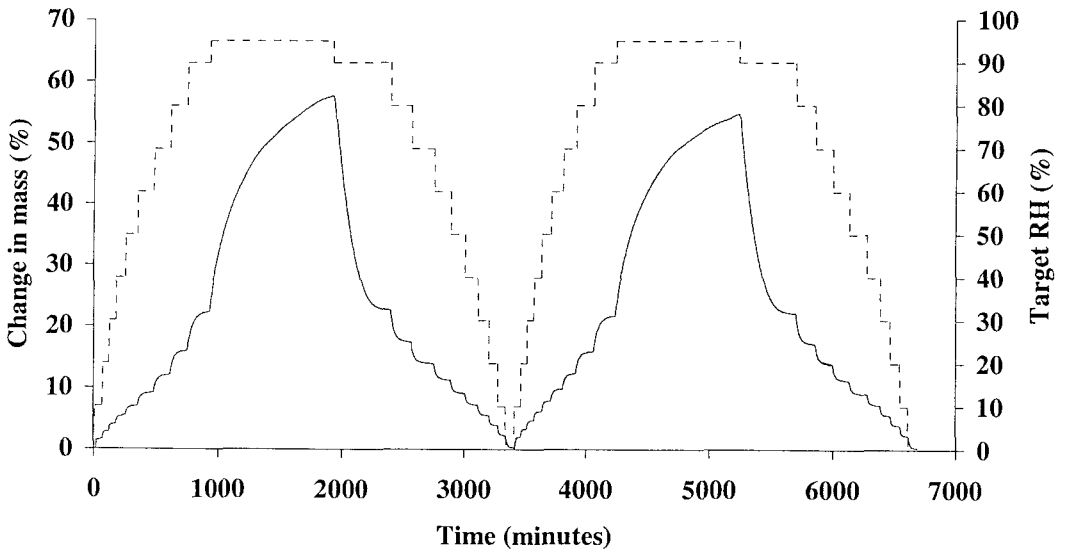


Figure 3.5 **DVS trace obtained on analysis of K4MP powder.**
Broken line shows target RH, continuous line shows percentage change in mass.

3.3 Results and Discussion

3.3.1 Distribution of insulin in lyophilised nasal inserts

Distribution of insulin in the different nasal insert formulations was found to be close to that which would theoretically be expected assuming dose uniformity. However, the total insulin content of each of the lyophilisates studied, was consistently higher than that which would be theoretically expected.

The reason for this apparent discrepancy between theoretical and experimentally derived insulin content is undetermined, although the fact that the difference remains relatively constant suggests that some sort of experimentally induced factor may be responsible.

However, it is clear from the results obtained that no change in the overall distribution of insulin occurs, either during the overnight storage of the gel or during the lyophilisation process.

3.3.2 *In-vitro* release of insulin from lyophilised nasal inserts

Release profiles from the insulin loaded lyophilised inserts are shown in Figure 3.6. The rate of insulin release from the lyophilised preparations decreased in the order 0.5% K4MP > 1% K4MP > 2% K4MP > 3% K4MP, and it can be observed from the profiles that for all of the lyophilised formulations, little or no insulin release occurs until 30 minutes.

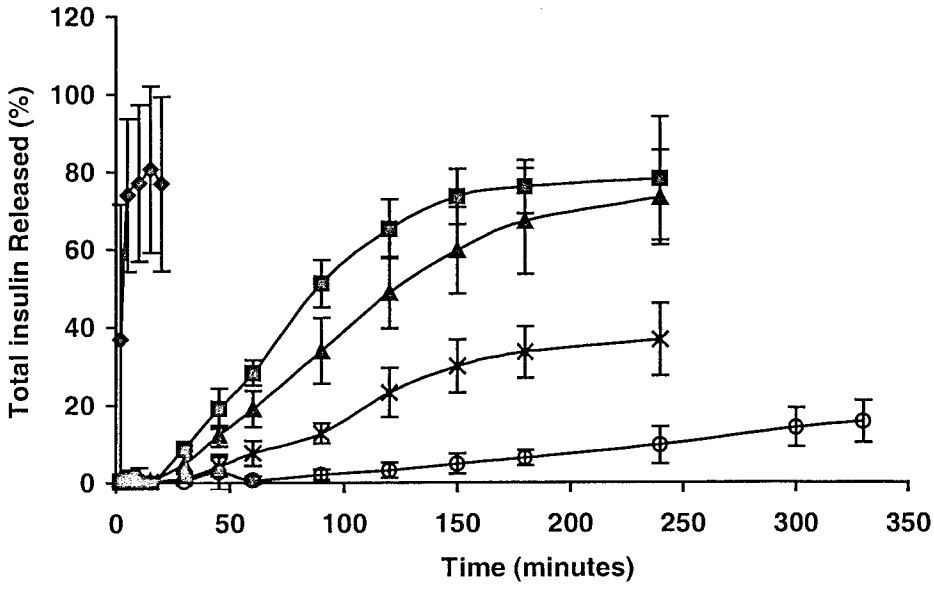


Figure 3.6 Profiles showing release of insulin from formulations containing 49iu insulin.

(◆) insulin solution, (■) 0.5% K4MP lyophilised insert, (▲) 1% K4MP lyophilised insert, (x) 2% K4MP lyophilised insert, (○) 3% K4MP lyophilised insert.

From the profiles displayed it is also evident that for the lyophilised preparations an initial linear phase of release occurs (Figure 3.7), following which the rate of release begins to slow. The exception to this is the 3% K4MP formulation, which for the time period studied (330 minutes) continued to show linear release. The profiles obtained may be evidence of the large molecular weight of the insulin molecule hindering its diffusion through the hydrated HPMC matrix, resulting in a release profile where this is the rate limiting step. This linear phase of release was not observed in a previous study where the release of NHT from lyophilised HPMC nasal inserts was examined (Thapa et al., 1999a). In this study a curved release

profile was observed, which perhaps reflects the water soluble nature of the NHT producing a different release profile.

A similar release profile has been reported in a study on the release of protein from dextran methacrylate (DM) hydrogels (Meyvis et al., 2001), where initial zero order release was followed by a slowing of release rate. The authors concluded that the release of protein from the matrix in hydrogels with a high degree of DM substitution was controlled by the size of the protein molecule. The slope of the linear phase of release shows good correlation with the initial hydration rates observed in the LSCM study in Chapter 2 (Figure 3.8), suggesting that there may be a connection between the rate of hydration of the lyophilised insert and the rate of insulin release. Other researchers have made similar observations, concluding that polymer hydration was an important factor in the mobility of large molecular weight proteins (Simon et al., 1999). This was not the case in a study of drug release from HPMC matrices (Sako et al., 2002), where it was found that while water penetration into the matrix was enhanced by the addition of water soluble excipients, drug release profiles were not affected. However, there was found to be an *in-vivo* effect. It has been reported that for HPMC systems there are a number of important factors involved in drug release, including hydration, swelling, water solubility of drug and chain length and degree of substitution of the HPMC polymer used (Siepmann and Peppas, 2001).

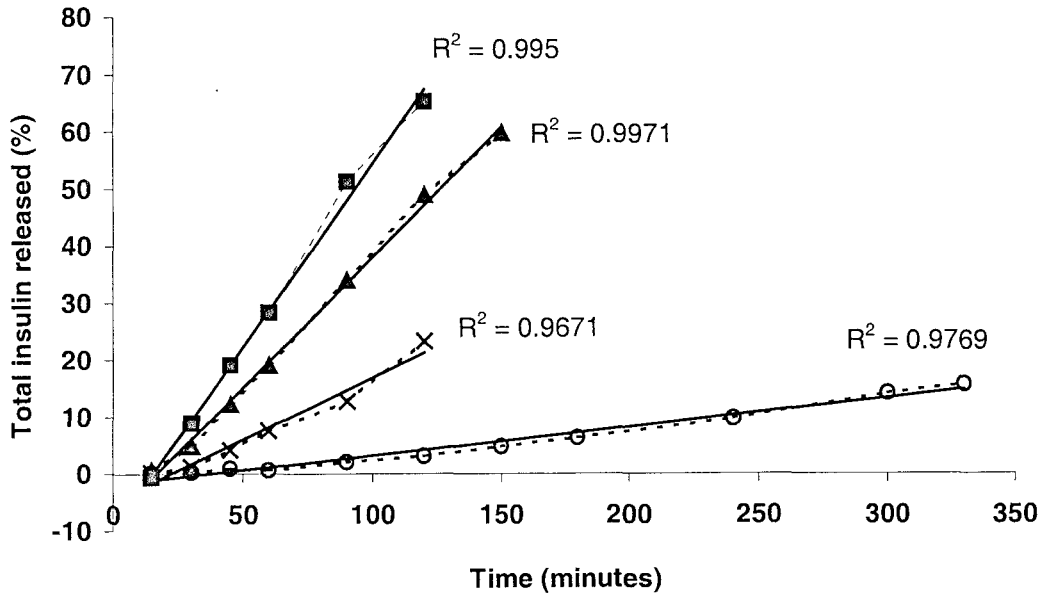


Figure 3.7 **Linear release phase of lyophilised inserts containing 49iu insulin.** (■) 0.5% K4MP lyophilised insert, (▲) 1% K4MP lyophilised insert, (x) 2% K4MP lyophilised insert, (○) 3% K4MP lyophilised insert.

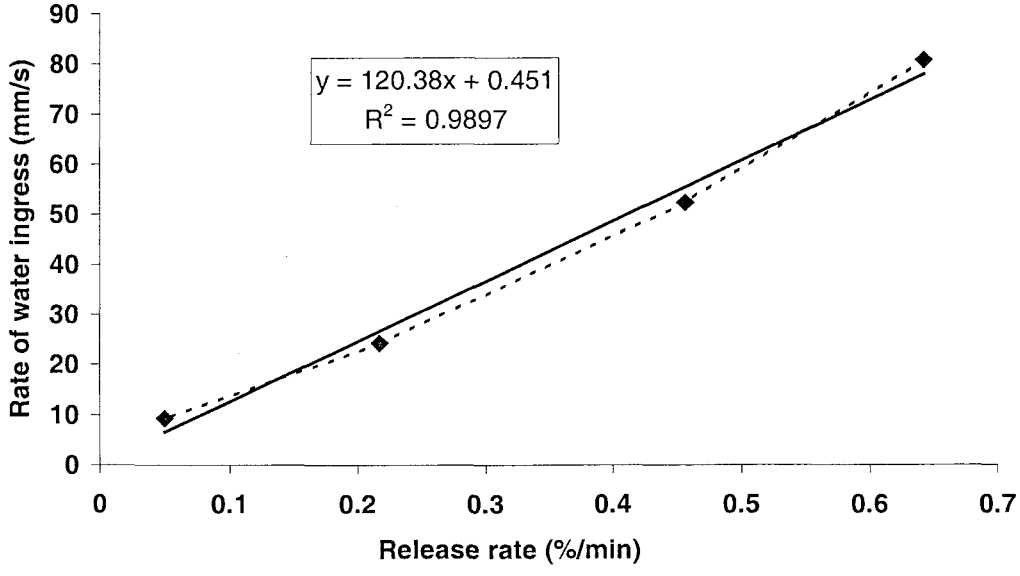


Figure 3.8 **Correlation between rate of linear phase of insulin release (x-axis) and initial rate of water ingress determined by the LSCM (y-axis).**

Subsequent to this, as the concentration of insulin in the hydrated matrix is reduced, the release rate begins to fall. The profile for the 3% K4MP formulation may not in the time period studied have released enough insulin to have reached the threshold where the rate limiting step is no longer the hindrances of insulin diffusion through the matrix. The suggestion that matrix diffusion is responsible for the initial linear release rate is supported by the fact that the rate of release decreases as the K4MP concentration of the lyophilisate increases, along with the observation that the simple insulin solution does not display this behaviour.

The maximum total amount of insulin released from the lyophilisates over a period of 4 hours was around 80% of the concentration of insulin contained within the 0.5% K4MP formulation. The total amount of insulin released further reduced as K4MP concentration increased. However when the insulin solution was studied using the dissolution apparatus, the maximum total quantity of insulin measured was also only approximately 80% that of the expected value. It may be possible that a proportion of the insulin becomes trapped in the filter paper which was used as a membrane for the dissolution test. This was briefly studied by leaving the used filter paper membrane in fresh PBS overnight, and then testing the solution for the presence of insulin. It was found that insulin which had presumably previously been located in the filter paper was present in the PBS solution. It can be seen from Figure 3.7 that the 3% K4MP lyophilisate released very little of its insulin content over a 330 minute period. The 0.5% and 1% K4MP formulations gave a similar final percentage release of insulin, although initially the rate of release was more rapid for the 0.5% formulation. Similar results were also found in the study by (Meyvis et al., 2001), with the authors suggesting that protein remains trapped within the hydrogel.

When 2% K100LV polymer was used instead of 2% K4MP, the insulin release profiles were statistically very similar (Figure 3.9), with overall only one time point showing statistically significant difference between the two. However, despite this similarity, the 2% K100LV insert does not display the initial linear release as observed with the K4MP formulations. Instead, a curved profile is produced, similar to that previously observed for NHT release (Thapa et al., 1999a). This may be explained by the lower molecular weight of the K100LV polymer producing less of

an obstruction to insulin diffusion through the matrix, so that it is not the rate limiting step but a part of the overall process defining insulin release.

Ultimately, the 2% K100LV lyophilisate released slightly less insulin than the 2% K4MP formulation, which is of significance as the 2% K4MP lyophilisate was shown in the adhesion tests of Chapter 2 to be highly superior to the 2% K100LV formulation. This means that while the 2% K100LV formulation may display adequate *in-vitro* release of insulin, it would be unlikely to have the same effect as 2% K4MP *in-vivo* due to its lack of adhesive properties.

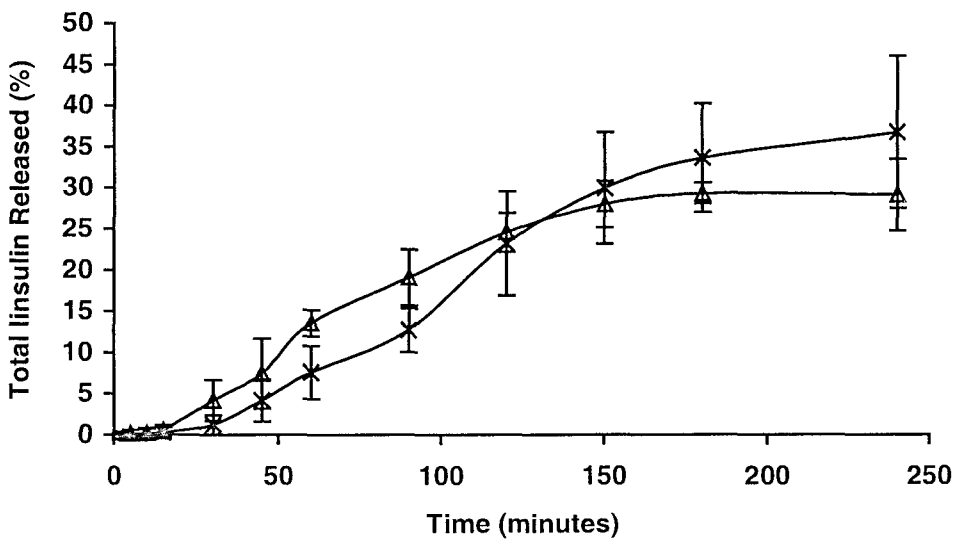


Figure 3.9 Profiles showing release of insulin from lyophilised formulations containing 49iu insulin.

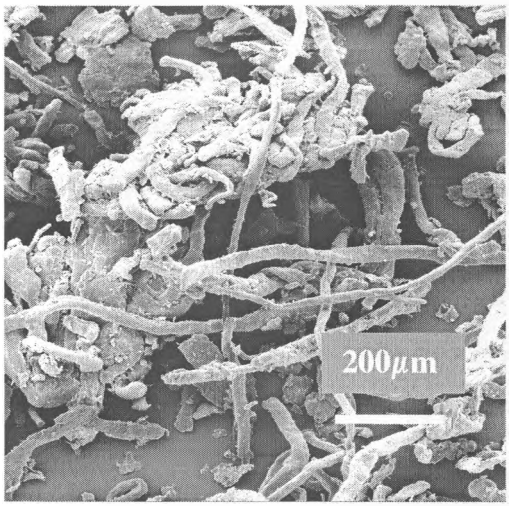
(×) 2% K4MP, (△) 2% K100LV.

3.3.3 Scanning electron microscopy

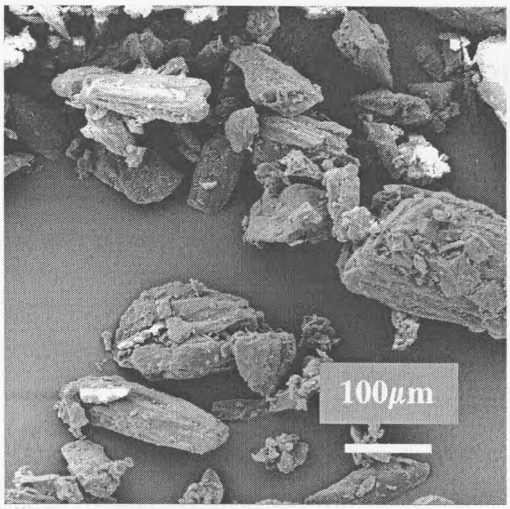
When examined with the SEM, it was observed that the internal structure of the HPMC lyophilisate was entirely different from the structure of either the polymer or mannitol powder from which it was formulated. Figure 3.10 shows an SEM image of K4MP powder, with the tangle of polymer strands of varying sizes clearly visible, and also the crystalline nature of the mannitol powder which is incorporated into the formulation.

Figure 3.10 shows that the resultant lyophilisate of a 2% K4MP gel (with 1% mannitol) displays none of the physical characteristics of the two original constituents. It can be seen that the process of dissolving the HPMC polymer and then lyophilising the resultant solution has altered the physical formation of the compounds, a sponge-like, highly porous internal structure being clearly visible. Assuming that the porous structure observed is formed from the HPMC, the mannitol which was contained within the lyophilised formulation does not appear to be evident in Figure 3.10, as there is no trace of the mannitol crystals amongst the K4MP polymer, and it may be possible that the mannitol has become incorporated into the HPMC structures. Closer examination of a lyophilisate prepared from 2% K4MP alone shows that the HPMC structure has changed in appearance, a smooth surface evident compared with the tangled, uneven nature of the polymer strands prior to freeze-drying.

The honeycomb structure formed is most likely the result of the removal of ice crystals during the sublimation process of lyophilisation, and the pores observed would correspond to the space previously occupied by ice crystals following the freezing process.



(a)



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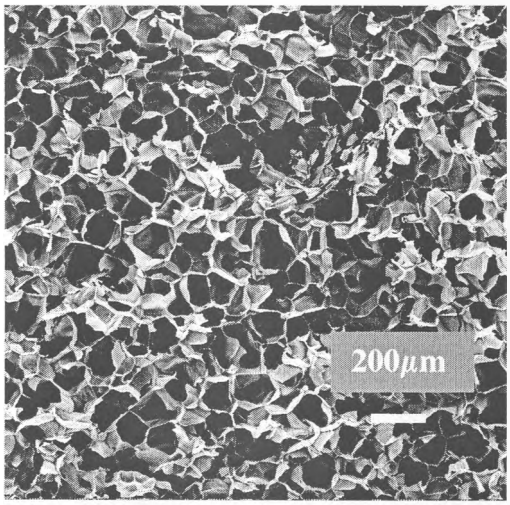

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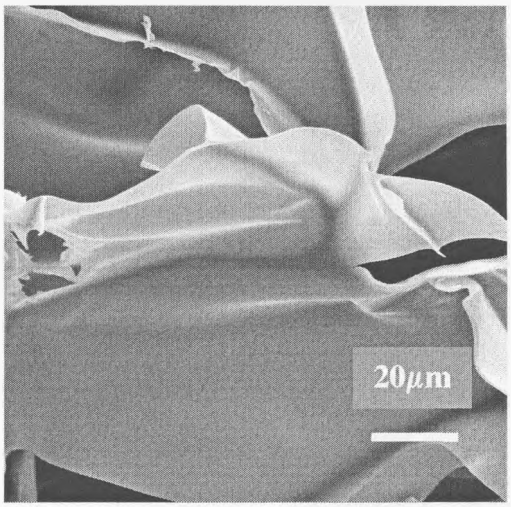


(d)

Figure 3.10 SEM images (1).

(a) K4MP powder, showing polymer strands, (b) mannitol powder, showing crystalline material, (c) an internal section of a 2% K4MP/1% mannitol lyophilisate, demonstrating a highly porous internal structure, and (d) the smooth surface of lyophilised K4MP (2% with no additives).

The average pore size of lyophilisates containing varying concentrations of K4MP polymer (with mannitol), and on addition of NHT and insulin to the formulation was calculated and results are shown in Table 3.1. Increasing the K4MP concentration results in a decrease in the average internal pore size of the lyophilisate, suggesting that during the freezing process the higher HPMC concentration forces the water contained within the gel to form smaller ice crystals. This effect is not linear however (Figure 3.11), and shows signs of plateauing at the 3% K4MP concentration, suggesting that this concentration is near a point where the pore size no longer decreases with increasing HPMC concentration.

Table 3.1 Effect of lyophilisate content on average internal pore size.

K4MP concentration	Drug content	Average pore size ± s.d. (μm)
0.5%	-	164.3 (±30.0)
1%	-	132.1 (±31.4)
2%	-	91.14 (±25.7)
3%	-	75.00 (±18.6)
2%	2mg nicotine	64.29 (±14.3)
2%	49 iu insulin pH3	103.6 (±14.3)
2%	49 iu insulin pH7	107.1 (±27.1)
2%	(Post DVS)	78.60 (±8.57)

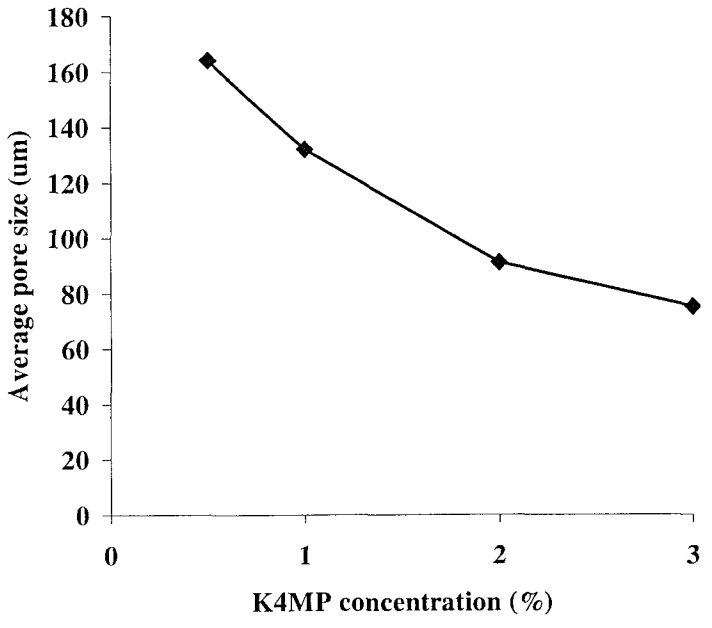


Figure 3.11 The effect of K4MP concentration on the internal pore size of lyophilised formulations.

The average pore size determined was found to produce good correlation with both the rate of insulin release during the linear phase (Figure 3.12), and the initial rate of water ingress found using CLSM (Figure 3.13), suggesting that these properties may be inter-related.

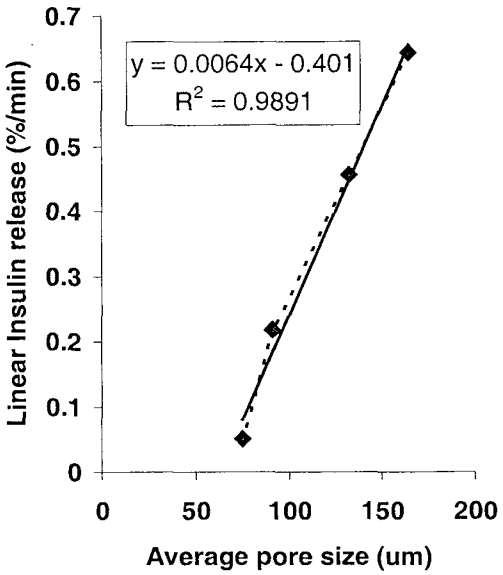


Figure 3.12 **Correlation between average pore size determined by SEM, and release rate of insulin during linear phase.**

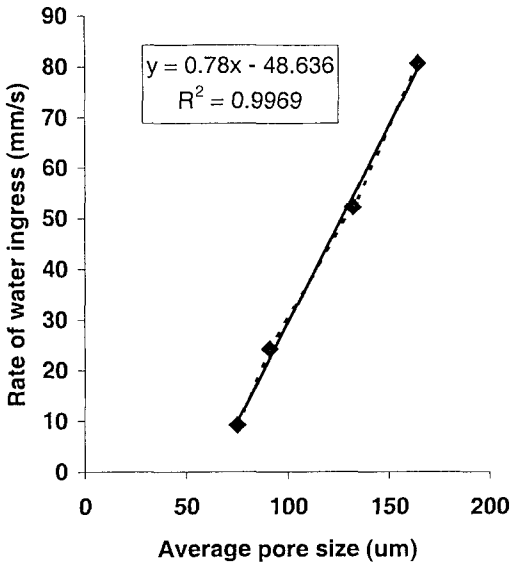


Figure 3.13 **Correlation between average pore size determined by SEM, and initial rate of water ingress using the CLSM.**

Incorporation of NHT results in a decrease in pore size, while addition of insulin formulated at either pH 3 or pH 7 increases the pore size of the lyophilisate in comparison with the placebo formulation. Examination of an insert which had previously been exposed to up to 95% RH in the DVS study (section 3.3.4) showed that exposure to high humidity reduced the internal pore size of the formulation. This may be a result of partial hydration of the lyophilisate due to exposure to water vapour, resulting in a partial collapse of the internal structure, and slight shrinkage upon drying at 0% RH.

Table 3.2 shows that lyophilisation of 2% K4MP alone (no mannitol) results in a decrease in the pore size of the lyophilisate compared to that which contains 1%

mannitol, which may further explain the CLSM finding in Chapter 2 that the rate of water ingress into 2% K4MP lyophilisates is lower in the absence of mannitol. Lyophilisation of 2% K4MP and NHT produces a pore size similar to that of the 2% K4MP/1% mannitol formulation, and higher than that observed with 1% mannitol in this K4MP/NHT combination (Table 3.1). Table 3.2 also shows that the addition of sodium fluoroscein to the K4MP (no mannitol) lyophilisate for CLSM hydration tests did not alter the pore size of the formulation.

The results appear to suggest that the addition of either mannitol or NHT to the K4MP lyophilisate produces an increase in the pore size of the formulation, while addition of both mannitol and NHT together results in a decrease in internal pore size. The cause of this effect remains unclear.

Table 3.2 The effect of the presence of mannitol, NHT and sodium fluoroscein on the internal pore size of lyophilisates of 2% K4MP.

Formulation	1% Mannitol	Average pore size ± s.d. (μm)
K4MP	Yes	91.14 (±25.7)
K4MP	No	84.78 (±26.2)
K4MP with NHT	No	92.94 (±27.0)
K4MP with Fluoroscein	No	85.86 (±30.3)

Examination of the internal structure of the lyophilisates at higher magnitude revealed that on the thin leaf like structures which surround the pores of the internal structure, evidence of the mannitol in the formulation could clearly be seen. In Figure 3.14 the 0.5% K4MP formulation shows clear evidence of a crystalline needle structure which has imposed itself on the K4MP leaves, thought to be the mannitol contained in the formulation which has transformed to a different crystal polymorph from that observed in the powder state (Figure 3.10). As the K4MP concentration is increased the presence and size of the crystalline mannitol structure becomes less evident. Presumably as the ratio of mannitol to polymer is decreased, so the mannitol becomes less able to impose its crystalline structure on the K4MP.

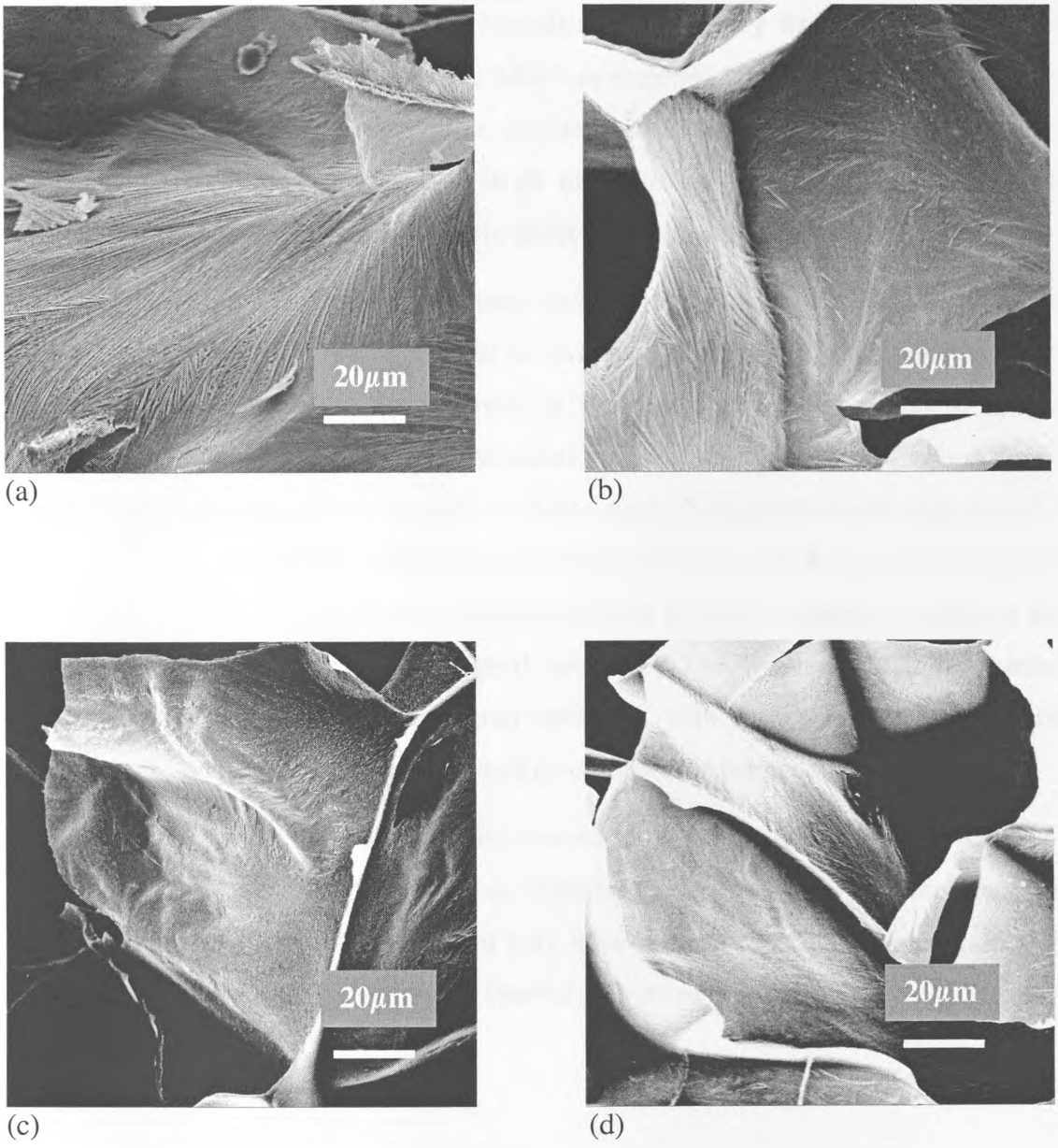


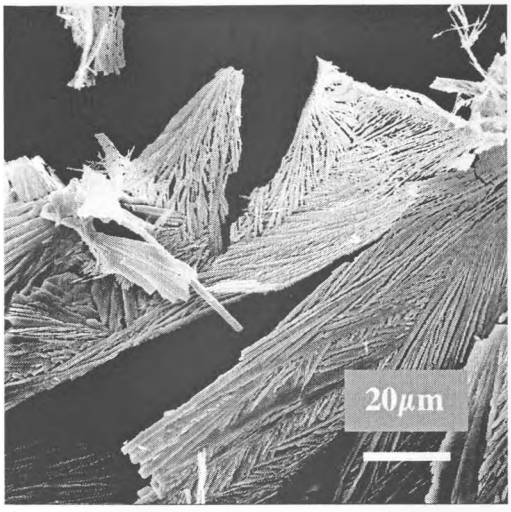
Figure 3.14 SEM images of lyophilised formulations.

Lyophilised (a) 0.5% K4MP, (b) 1% K4MP, (c) 2% K4MP and (d) 3% K4MP, each with 1% mannitol.

The images shown in Figure 3.15 demonstrate the crystal structures produced on lyophilisation of 1, 5, 10, and 20% mannitol solutions. The lyophilised 1% mannitol solution shows a needle like crystal structure which is very similar in both size and formation to the crystalline structure which is imposed on the HPMC polymer of a lyophilised 0.5% K4MP formulation, as shown in Figure 3.14. Lyophilisation of a 5% mannitol solution produced crystals of a similar shape to the 1% mannitol solution, although they were of slightly different proportions, longer and wider.

Lyophilised 10% mannitol solution results in crystals which appear partially needle like, although there appears to be evidence of a triangular shaped formation occurring, suggesting that the mannitol is forming a different polymorph to that observed with lyophilised 1% and 5% solutions, or a amalgamation of two different polymorphs. This is consistent with a study which observed that slow freezing of a 10% w/v mannitol solution produced a mixture of the α and β crystal polymorphs (Kim et al., 1998). The crystal structures formed are more compact, reflecting the increasing concentration of the original solution. The lyophilised 20% mannitol solution shows an even more compact structure, with the triangle shaped crystal polymorph predominantly displayed, and no evidence of the needle formation.

These images demonstrate that the concentration of mannitol which is present upon lyophilisation appears to have an important effect on the crystalline mannitol polymorphs which are formed, which may have an influence on the preparation of some formulations, depending on the desired properties of the product.



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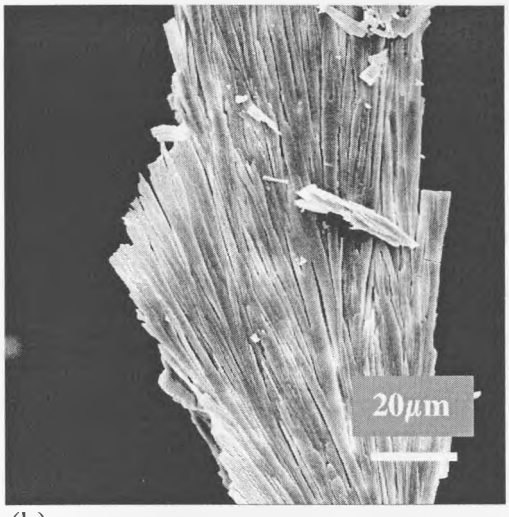

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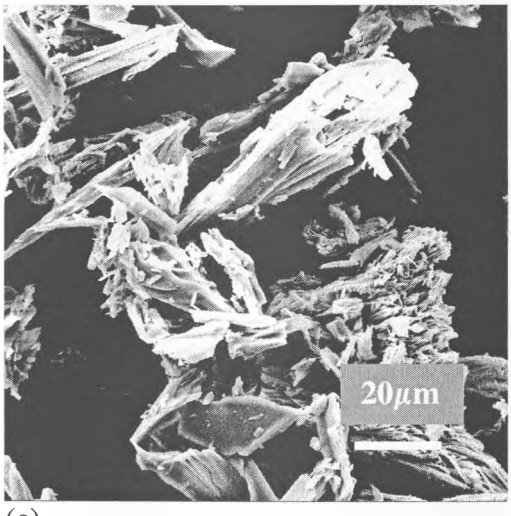

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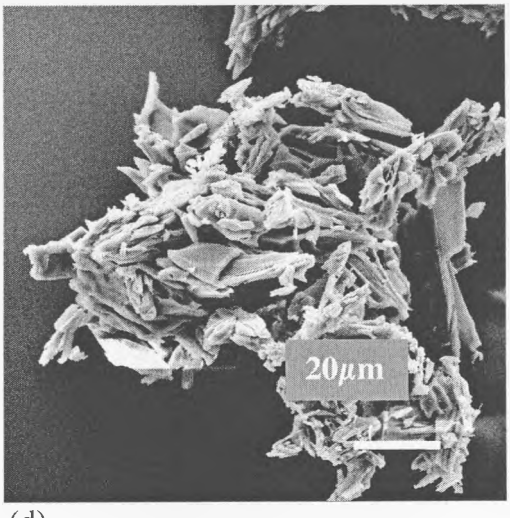


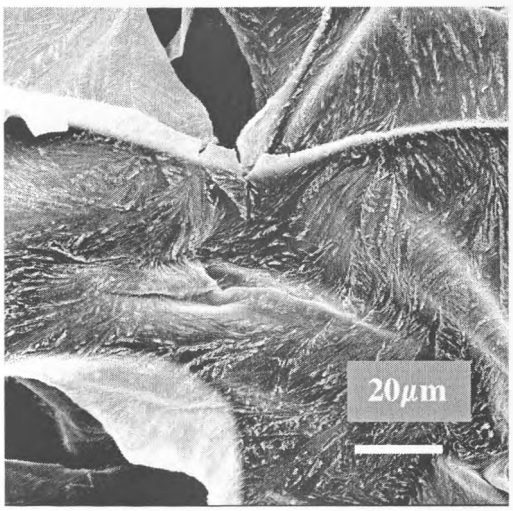
(d)

Figure 3.15 SEM images of freeze dried mannitol.

Freeze dried from solutions of (a) 1% mannitol, (b) 5% mannitol, (c) 10% mannitol and (d) 20% mannitol.

Figure 3.16 shows the effect that the addition of insulin to a 2% K4MP lyophilisate makes, depending on the pH of the preparation prior to lyophilisation. Evidence of a crystalline structure superimposed on the HPMC leaves is still visible, however in the formulation prepared at pH 3, the crystalline structures are slightly larger and more prominent than for the 2% K4MP placebo formulation. The insulin containing insert prepared at pH 7 shows a slightly smaller crystalline structure, more similar to that of the placebo 2% K4MP formulation, which would have been prepared at a similar pH. It is unclear if the inclusion of insulin also affects the structure observed here, however, from the image of insulin powder prior to lyophilisation it can be observed that the native formation of the insulin powder has been altered during dissolution and subsequent lyophilisation.

Figure 3.16 also shows an SEM image of a 2% K4MP placebo formulation, which has been exposed to a full DVS cycle as described in section 3.2.3.6. The exposure of the formulation to high levels of humidity has resulted in a change in the mannitol structure in the formulation, which was faintly visible prior to exposure to water vapour, to a larger, more defined crystal structure. This could be due to crystalline mannitol present becoming hydrated by the moisture in the water vapour, aggregating, and reforming a different crystal polymorph structure upon drying. This occurrence has implications for the storage and stability of the formulation. It is clear that such lyophilised formulations containing mannitol must be maintained in dry conditions to avoid this change in structure where possible, in order to avoid any adverse effects on the stability of the product, and the drug contained within.



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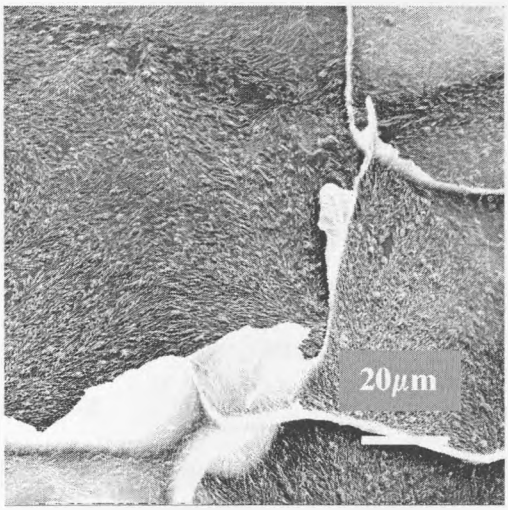

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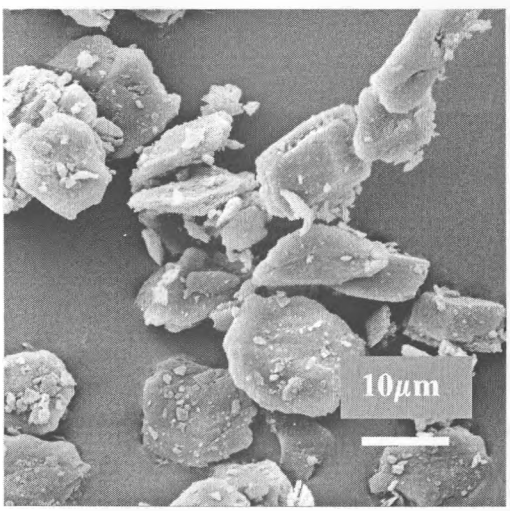

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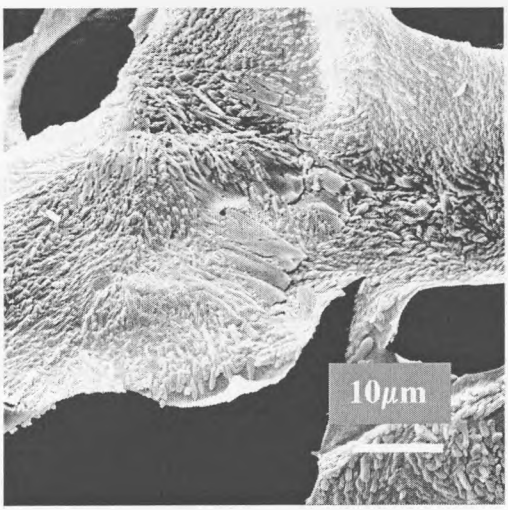


(d)

Figure 3.16 SEM images (2)

Showing the structure of (a) lyophilised 2% K4MP inserts on addition of insulin at pH 3 (b) lyophilised 2% K4MP inserts on addition of insulin at pH 7, (c) insulin powder, and (d) a 2% K4MP/1% mannitol lyophilisate after exposure to up to 95%RH.

The image of a lyophilised 2% K4MP (with 1% mannitol) insert containing NHT in Figure 3.17 shows a different structure present on the HPMC "leaves" to any of the other formulations studied. The appearance of small clusters of material can be seen, suggesting that the addition of NHT has in some way altered the structure which is formed. The clusters observed do not appear ordered, as in the other SEM images, although some evidence of crystalline portions can be seen. The existence of these small clusters of material may be attributable to the presence of NHT in the formulation forming these structures in conjunction with the mannitol. Alternatively, it may be possible that these structures are due to the formation of a partially amorphous/partially crystalline mannitol structure.

It is clear that the structures observed bear no resemblance to the images of NHT powder, crystalline in nature before freeze drying, and forming cocoon-like pod structures after freeze drying of a solution (Figure 3.17). Lyophilisation of K4MP and NHT in the absence of mannitol does not result in the appearance of the cluster structures, nor a crystalline formation, but instead shows a brittle looking coating on the surface of the K4MP "leaves", with a similar texture to the surface of the freeze dried NHT "cocoons" (Figure 3.17). The lack of the presence of cluster structures in the lyophilisate of K4MP and NHT in the absence of mannitol suggests that it may be the mannitol in the formulation, rather than NHT, which is involved in their formation.

In gathering the SEM images which have been displayed in this chapter, occasional observations of such small cluster like formations were also made amongst the general crystal structures which are imposed on the HPMC in placebo K4MP/mannitol lyophilisates, further indicating that it may be the mannitol in the formulation, and not NHT, which forms these clusters.

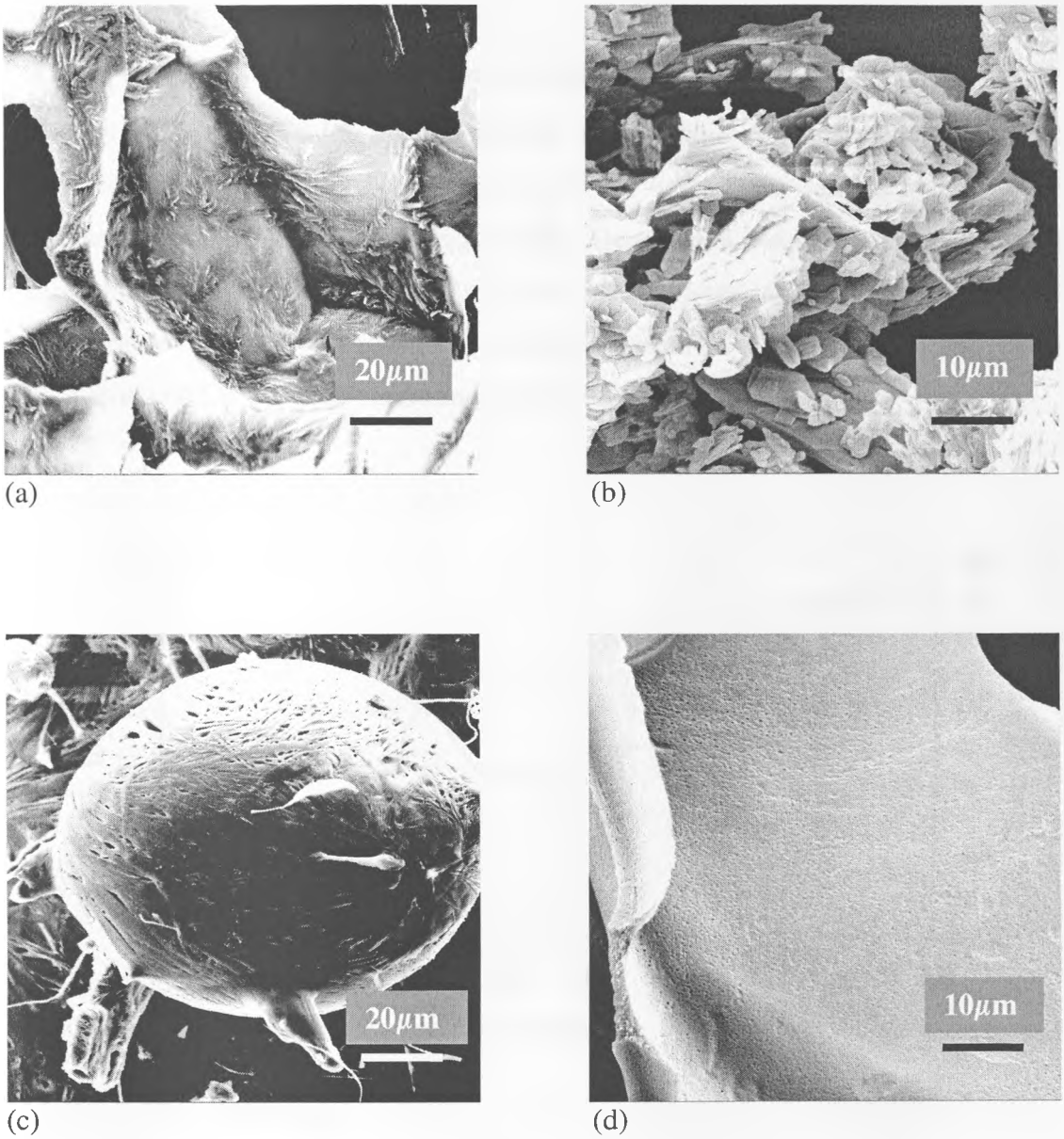


Figure 3.17 SEM images (3)

Showing (a) cluster structures present on the surface of a lyophilisate of 2% K4MP/1%mannitol and NHT, (b) NHT powder crystals prior to lyophilisation, (c) NHT cocoon like structure formed from a freeze dried NHT solution, and (d) the brittle appearance of the surface of an HPMC "leaf" of a lyophilisate containing 2% K4MP and NHT in the absence of mannitol.

3.3.4 Dynamic vapour sorption

3.3.4.1 General observations and recrystallisation event

The DVS analysis for the lyophilised formulations demonstrated a substantial increase in weight as a result of sorption of water vapour, particularly in the phase of exposure to 95% RH (shown in Figure 3.5). However, a weight loss event occurring during the sorption phase of the first cycle was observed, at approximately 50-80% RH (Figure 3.18), similar to data previously reported for amorphous lactose powder (Buckton and Darcy, 1995, Lane and Buckton, 2000).

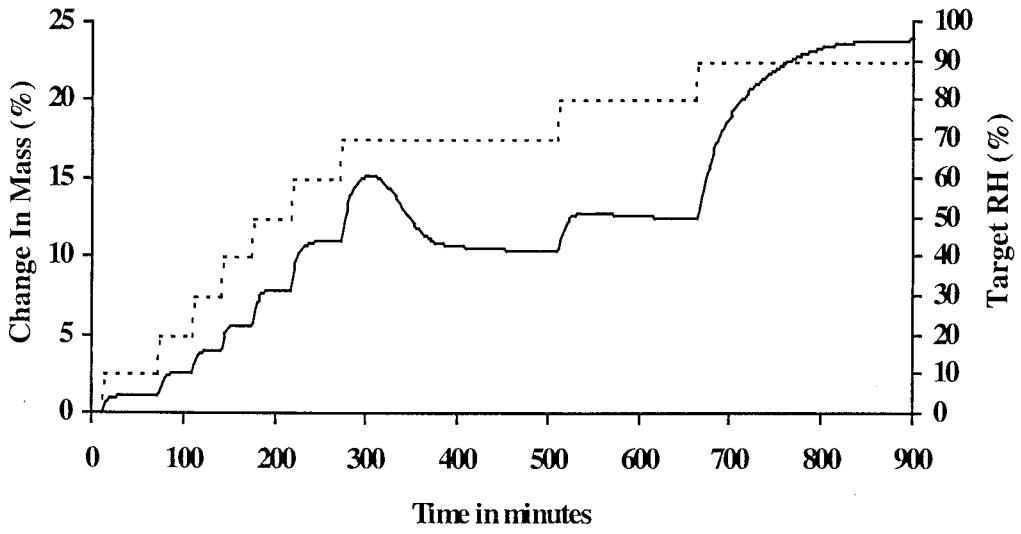


Figure 3.18 DVS trace.

Showing weight loss event in a lyophilised nasal insert (containing 2% K4MP/1% mannitol and NHT), occurring following increase in RH to a critical level. Continuous line shows change in weight as a percentage of the dry weight of the sample, broken line shows relative humidity.

The effect is thought to be a consequence of water vapour sorption, which by plasticising an amorphous component (apparently lyophilised mannitol) and lowering the glass transition temperature (Tg) to below room temperature, triggered a transformation to a crystalline form. During the second cycle this effect was not seen, and this absence of weight loss would be explained by the irreversibility of crystallisation at room temperature.

Mannitol is known to exist as different crystal polymorphs, α, β and δ, and transitions between these polymorphs have been observed on exposure to 97% RH (Yoshinari et al., 2002). Results reported by (Kim et al., 1998) suggest that there is a threshold concentration of 30% w/w above which mannitol is detected in a crystalline state, when lyophilised with a noncrystallising solute. As the concentration of mannitol in the solutions prepared for lyophilisation here are below this threshold, this may explain the apparent presence of amorphous material in the lyophilised inserts. It has also been reported that rapidly cooled 20°C/min) solutions of 10% w/w mannitol remain amorphous, and that slower cooling rates (5 or 10 °C/min) induced partial crystallisation (Pyne et al., 2002), although the rate of freezing in experiments reported in this work are much slower.

The RH at which the recrystallisation initially occurs (the event may sometimes continue over a range of increasing RH) appears to be dependant on both the HPMC and mannitol concentration of the formulations (Table 3.3). As K4MP concentration is increased the RH of the recrystallisation event decreases, while conversely, increasing mannitol content results in an increase in the RH at which the event occurs.

Table 3.3 Effect of formulation variables on RH at which recrystallisation is initially observed in lyophilisates containing K4MP, mannitol and NHT (equivalent to 2mg nicotine base).

Formulation variable concentration		RH weight loss seen
K4MP	Mannitol	
0.5%	1%	80%
1%	1%	70%
2%	1%	60%
3%	1%	50%
2%	0.5%	50%
2%	1%	60%
2%	2%	70%
2%	3%	70%

3.3.4.2 Sorption analysis of powders

The total amount of water vapour sorbed by each powder varied greatly (Table 3.4). Mannitol powder sorbed very little moisture, 0.30% of its dry weight, whereas K4MP powder sorbed over half of its dry weight in water vapour. The lower molecular weight polymer, K100LV, was only capable of absorbing less than 60% of the amount of water vapour that K4MP sorbed.

DVS analysis of the individual components of each of the formulations shows that this crystallisation event is not evident in any of the original powders, apart from the mannitol, which displayed a very slight weight loss due to recrystallisation. The weight loss was negligible, showing that the mannitol powder was, as expected given the SEM images obtained, essentially crystalline in nature. The lack of any crystallisation event in any of the individual powders suggests that it is lyophilisation that induces the formation of the amorphous material.

Table 3.4 Vapour sorption analysis of individual powders.

Powder	H_2O sorbed*	H_2O lost during crystallisation*	μmol H_2O lost per μmol mannitol
K4MP	57.65	-	-
K100LV	32.69	-	-
Mannitol	0.30	0.01	0.00
NHT	19.40	-	-
Insulin	21.93	-	-

*expressed as a percentage of the dry weight of the formulation.

3.3.4.3 Sorption characteristics of lyophilised solutions

Lyophilisation of solutions of the powders (Table 3.5) has little effect on the behaviour of K4MP polymer (lyophilised as a 5% solution), which sorbed over half its own weight in water vapour. Lyophilisation of a NHT solution (2mg nicotine base/0.26ml) however, doubles the total sorption capacity of NHT powder.

Lyophilisation of mannitol solution produces different results according to the concentration of the solution. A lyophilised 1% mannitol solution has increased the sorption capacity of mannitol powder by a factor of 30, and no crystallisation effect is observed. Lyophilised 5% and 10% mannitol solutions had reduced sorption capacities compared to the 1% solution, although here some evidence of a crystallisation effect was observed. Increasing mannitol content of the lyophilised solution to 20% gave a similar sorption capacity to the 5% solution, but there was no evidence of recrystallisation.

Table 3.5 DVS analysis of lyophilised solutions.

Lyophilised solution	H_2O sorbed*	H_2O lost during crystallisation*	μmol H_2O lost per μmol mannitol
K4MP (5%)	51.91	-	-
Mannitol (1%)	9.09	-	-
Mannitol (5%)	1.80	0.02	0.00
Mannitol (10%)	-0.01	1.26	0.13
Mannitol (20%)	1.75	-	-
NHT	45.05	-	-

*expressed as a percentage of the dry weight of the formulation.

3.3.4.4 Lyophilised placebo HPMC inserts

Co-lyophilisation of K4MP with mannitol further altered the sorption capacity and recrystallisation effect (Table 3.6). Sorption capacity for these formulations ranged between 25-37% of the dry weight, lower than found for K4MP alone, which may be a result of its combination with the lower sorption capacity mannitol. When calculated as mg H_2O sorbed per mg K4MP in the insert a good correlation was found (R^2 = 0.997), showing that the vapour sorption capacity is at least in part dependant on HPMC content. In fact, increasing K4MP concentration results in a decrease in sorption capacity. However, the DVS cycle used had a maximum step time of 999 minutes, and in many samples studied it was clear that this time maximum had been reached at the 95% RH step, suggesting that the formulation may be capable of sorbing further quantities of water vapour if allowed.

The data in Table 3.6 also shows that a relatively high level of recrystallisation is observed, which when calculated as μmol H_2O lost per μmol mannitol is far greater than was observed in mannitol alone, apart from the lyophilised 10% solution of which it is in a similar range. The results suggest that the admixture with HPMC gave rise to a higher amorphous mannitol content, which resulted in an increased weight loss during the first cycle. From the current results it seems clear that the

presence of HPMC in the formulation influences the conversion of amorphous mannitol to the crystalline state, previously suggested by (Fakes et al., 2000).

No pattern in this effect was observed, aside from that in the case of both lyophilised mannitol solutions and lyophilised K4MP/mannitol solutions, it appears to be those in the "middle range" of concentrations studied which display the highest level of recrystallisation.

Table 3.6 Effect of co-lyophilisation of K4MP and 1% mannitol on DVS analysis results.

K4MP	H_2O sorbed*	H_2O lost during crystallisation*	μmol H_2O lost per μmol mannitol	μmol H_2O lost per mg HPMC
0.5%	25.83	0.004	0.063	0.692
1%	36.19	0.765	0.155	0.849
2%	37.02	0.710	0.216	0.592
3%	28.83	0.280	0.116	0.207

*expressed as a percentage of the dry weight of the formulation.

3.3.4.5 Lyophilised inserts containing NHT

Addition of NHT (equivalent to 2mg nicotine base) to the K4MP/mannitol formulations (Table 3.7) produced a large increase in both the sorption capacity and amorphous content of the lyophilisates. Samples were capable of absorbing between 75-120% of their dry weight in water vapour, excluding the 0.5% K4MP formulation, which had a relatively low sorption capacity of 6.9%. No correlation was observed here between vapour sorption and K4MP concentration, suggesting that this parameter is altered by the addition of NHT to the formulation. The extent of recrystallisation varied greatly, and when expressed as μmol H_2O lost per μmol mannitol, ranged from a 3-28% increase in comparison with the equivalent K4MP

placebo formulation. This increase in amorphous content may explain the presence of small clusters of material observed in the NHT containing insert when studied using SEM in section 3.3.2. The structures may be evidence of the amorphous material which is recrystallising, easily observed in images obtained for this formulation due to their increased presence. The infrequency of these SEM observations in inserts which do not contain NHT agree with the DVS data where the recrystallisation event was less pronounced.

The use of the low molecular weight K100LV polymer in place of K4MP produced effects which were of a similar magnitude in terms of both vapour sorption capacity and amorphous content. Despite these findings, it was still not possible to discern any patterns in the data in terms of excipient content, or H_2O loss during crystallisation.

Table 3.7 DVS data on lyophilised formulations containing HPMC, 1% mannitol and NHT (equivalent to 2mg nicotine base).

HPMC	H_2O sorbed*	H_2O lost during crystallisation*	μmol H_2O lost per μmol mannitol	μmol H_2O lost per mg HPMC
0.5% K4MP	6.9	4.25	0.98	10.8
1% K4MP	116	1.79	0.50	2.75
2% K4MP	107	5.21	1.99	5.47
3% K4MP	89.3	6.78	3.24	5.93
0.5% K100LV	120	2.16	0.50	5.45
1% K100LV	116	3.64	1.02	5.60
2% K100LV	99.2	1.86	0.51	5.65
3% K100LV	76.8	2.45	1.18	2.16

*expressed as a percentage of the dry weight of the formulation.

3.3.4.6 Reduction in sorption capacity

Table 3.8 demonstrates the reduction in sorption capacity in lyophilised formulations containing NHT (equivalent to 2mg nicotine base), following one complete DVS sorption/desorption cycle. On average this reduction is approximately 20% of the sorption capacity displayed in cycle one. This may be due to the recrystallisation of mannitol which occurs, or simply the exposure of the normally dry lyophilisate to high levels of humidity resulting in a partial collapse of the structure of the formulation, reducing its sorption capacity on subsequent exposure to humidity.

Table 3.8 Change in sorption capacity of formulations containing HPMC, 1% mannitol and NHT (equivalent to 2mg nicotine base) following one complete DVS sorption/desorption cycle.

HPMC	H_2O sorbed*	
	Cycle 1	Cycle 2
0.5% K4MP	62	26
1% K4MP	116	90
2% K4MP	107	89
3% K4MP	89	60
0.5% K100LV	120	104
1% K100LV	116	92
2% K100LV	99	79
3% K100LV	77	66

*expressed as a percentage of the dry weight of the formulation.

3.3.4.7 Reproducibility of data

Table 3.9 demonstrates that the results obtained from the DVS were generally reproducible, although on some occasions large standard deviations were observed, and the 3% K4MP formulation only displayed a recrystallisation event in one instance.

Table 3.9 Reproducibility of data from DVS experiments in formulations containing K4MP and 1% mannitol (n=6).

K4MP	H_2O sorbed*	H_2O lost during crystallisation*	μmol H_2O lost per μmol mannitol	μmol H_2O lost per mg HPMC
0.5%	26.05 ±1.08	0.354 ±0.38	0.074 ±0.04	0.818 ±0.39
1%	37.19 ±3.14	1.012 ±0.23	0.205 ±0.05	1.124 ±0.25
2%	42.80 ±5.09	0.772 ±0.07	0.234 ±0.02	0.643 ±0.05
3%	39.59 ±10.13	0.280	0.116	0.207

*expressed as a percentage of the dry weight of the formulation.

3.3.4.9 Effect of mannitol content

The effect of varying the mannitol content of the lyophilisates was studied in order to assess any effect on sorption capacity and amorphous content of the formulation (Table 3.10). No demonstrable change in sorption capacity was observed as a result of altering mannitol content, although an apparent effect on amorphous content can be seen. When the event is assessed as μmol H_2O lost in terms of μmol mannitol present, it can be seen that increasing mannitol concentration results in a decrease in the extent of amorphous content, although considering the lack of any discernable patterns in the rest of the data, this information should be treated with caution.

Table 3.10 **Effect of altering mannitol content of lyophilisates containing 2% K4MP and NHT (equivalent to 2mg nicotine base) on DVS analysis results.**

Mannitol	H_2O sorbed*	H_2O lost during crystallisation*	μmol H_2O lost per μmol mannitol	μmol H_2O lost per mg HPMC
0.5%	112.7	4.122	2.405	3.303
1%	107.0	5.210	1.990	5.470
2%	114.2	4.417	1.065	5.852
3%	98.0	3.103	0.604	4.974

*expressed as a percentage of the dry weight of the formulation.

3.3.4.10 Sorption capacity of lyophilised inserts containing insulin

The addition of insulin (bovine) to the K4MP/1% mannitol lyophilisates showed that when prepared at pH 3 the total amount of H_2O sorbed was of a similar magnitude to that of placebo K4MP formulations, whereas inserts formulated at pH 7 increased vapour sorption capacity (Table 3.11). There is no correlation between H_2O sorption and K4MP concentration in insulin containing inserts formulated at either pH 3 ($R^2 = 0.7765$), or pH 7 ($R^2 = 0.8755$), showing that in a similar manner to the data concerning NHT, the inclusion of insulin in the formulation alters sorption properties. This may be a result of the insulin content in the formulation being present in a different conformation due to the effects of pH on its structure. It has been reported (Costantino et al., 1998b) that lyophilisation of protein and sugar mixes lower the water monolayer (a level of hydration above which lyophilised proteins show increased stability), suggesting that an interaction occurs which reduced the availability of water binding sites.

For both formulations of insulin lyophilisates it can be seen that an increase in K4MP concentration results in a decrease in amorphous mannitol content, with higher concentration K4MP lyophilisates displaying no recrystallisation whatsoever.

This may be the result of a similar effect to that found by (Costantino et al., 1998b), who reported that in co-lyophilised sugar/protein mixes, the RH required for crystallisation to occur was higher than in the amorphous sugar alone, or did not occur at all.

Table 3.11 DVS analysis of lyophilisates containing K4MP/ 1% mannitol and 49iu bovine insulin formulated at pH 3 and pH 7.

K4MP/ pH	H_2O sorbed*	H_2O lost during crystallisation*	μmol H_2O lost per μmol mannitol	μmol H_2O lost per mg HPMC
0.5%/pH3	19.16	1.982	0.432	4.752
1%/pH3	25.41	1.520	0.408	2.244
2%/pH3	27.04	0.763	0.282	0.775
3%/pH3	33.33	-	-	-
0.5%/pH7	48.68	0.205	0.045	0.490
1%/pH7	48.03	0.083	0.022	0.122
2%/pH7	51.14	-	-	-
3%/pH7	35.16	-	-	-

*expressed as a percentage of the dry weight of the formulation.

3.4 Conclusion

Insulin release from the lyophilised formulations studied decreased in the order > 0.5% K4MP > 1% K4MP > 2% K4MP > 3% K4MP. The insulin release data shows an initial linear phase of release, which is dependant on K4MP concentration, and shows a correlation with the initial hydration rates determined using LSCM in Chapter 2. This linear phase is followed by a decrease in the release rate, following which the maximum insulin release obtained was 80% of the content of the insert.

A combination of SEM images and DVS data provided information on the effect of exposure of the lyophilised formulations to moisture in the form of water vapour. A crystallisation event was observed, thought to be due to the presence of amorphous mannitol recrystallising on exposure to water vapour. As the recrystallisation is due to the presence of water vapour lowering the glass transition temperature (Tg) of the mannitol to room temperature to allow crystallisation to occur, this will have implications for the storage conditions of the formulation. Depending on the concentration of the respective excipients, the room humidity (generally 60%) may induce this crystallisation event.

Chapter 4 *In-vivo* Evaluation of Lyophilised Nicotine Nasal Insert in Sheep

4.1 Introduction

Attempts to assess potential nasal absorption of a drug from a formulation have resulted in many researchers reporting data from *in-vitro* techniques which have been developed to simulate *in-vivo* mucosal transport. Such methods include the assessment of permeation across monolayers of Caco-2 cells, a human intestinal cell culture, which has been used as a model epithelial cell layer (Artursson et al., 1994, Behrens et al., 2002), and the use of cultured human nasal epithelial cells (Agu et al., 2002, Agu et al., 2001, Kissel and Werner, 1998). Also investigated has been the use of excised material such as porcine (Osth et al., 2002) or bovine (Schmidt et al., 2000) nasal mucosa in a horizontal Ussing chamber designed to mimic the air:mucosal surface interface. In one of these studies (Behrens et al., 2002) a correlation was found between the *in-vitro* cell culture model and rat epithelial cells. Despite the benefits of using such *in-vitro* methods, such as low cost and rapid results, ultimately it is necessary to examine a formulation *in-vivo*, where accurate pharmacokinetic information can be obtained.

Animals which have been used to obtain *in vivo* data on nasal drug absorption and pharmacokinetics include rats (Jung et al., 2000, Raghavan and Abimon, 2001, Tengamnuay et al., 2000, Arnold et al., 2002), rabbits (Ugwoke et al., 2000b, Bechgaard et al., 1999, Lim et al., 2002) and sheep (Illum et al., 1990, Illum et al., 2001a). Each animal model has advantages and disadvantages, which must be considered in the choice of the animal used. Rats are the most commonly reported animal model, as they are easily bred, economical to maintain and dose (only small quantities of drug will be required), and are easily handled. However, the small rat nasal cavity can make dosing difficult, and the widespread use of anaesthetic agents

reported may result in a falsely high value for bioavailability being obtained due to inhibition of the mucociliary function by the anaesthetic (Illum, 1996).

In animals the sense of smell is often much more important than in humans and as a consequence a more complex nasal structure has evolved (Illum, 1996). In addition, the septum which divides the two cavities of the human nose is incomplete in many animals, allowing access to one side of the nasal cavity from the other in the posterior area of the nose (Illum, 1996). Rabbits are useful for nasal dosing, as they are easily handled, dosed and bled, and are relatively economical to house. However the nasal anatomy of the rabbit is more similar to that of the dog than man (Gizurarson, 1990), and a large nasal surface area per Kg bodyweight is likely to result in high absorption (Illum, 1996).

The sheep is not often used in nasal studies despite the fact that the animal is also easily handled, dosed and blood sampled. This is probably due to the fact that they are relatively more expensive to keep and require larger housing facilities. The sheep has a nasal surface area per Kg that is similar to that of humans compared to other animals. For blood sampling a cannula can easily be placed in the jugular vein and maintained usually for at least 9-10 hours. Samples of 5-10ml can be taken on each occasion (Illum, 1996).

The volume of the nasal cavity varies from 0.4mL in rats, 6mL in rabbits, 20mL in humans and 114mL in sheep (Illum, 1996, Gizurarson, 1990). Nasal mucosal surface area per Kg of bodyweight varies between 41.6 for rats, 20.3 for rabbits, 2.5 for humans and 8.2 for sheep, showing that the size of the nasal cavity does not necessarily reflect the area available for absorption, nor the resultant dosage per Kg of bodyweight. In terms of nasal surface area, the volume of drug required in order to be equivalent to 13mL in the rat would be 58mL in the rabbit, 150mL in humans and 307mL in sheep (Gizurarson, 1990). The clearance half-life of a substance varies between 5 minutes in rats, 10 minutes in rabbits, 15 minutes in humans and 42 minutes in sheep (Gizurarson, 1990).

Other factors which may influence the outcome of animal experiments include enzymes, which may be present in the nose and metabolise the drug in question (for

example peptide drugs) (Illum, 1996), and the length of the nasal cavity (Gizurarson, 1990).

It is important to bear in mind that the nasal cavity of an animal is anatomically and physiologically different from humans, as a result of the different functions that the nose performs in animals. These differences must be remembered when attempting to use animal models to predict nasal absorption in humans. Published studies have offered conflicting evidence of the correlation of nasal absorption between rabbits, sheep and humans. Nasal bioavailability of buprenorphine was higher in both rabbits and sheep than in humans (Lindhardt et al., 2001), while administration of diazepam resulted in bioavailability which decreased in the order rabbits > humans > sheep (Lindhart et al., 2002). Another study however, concluded that the clearance rate of a bioadhesive chitosan system in sheep was comparable with that found in humans, making the sheep a good predictive model for humans (Soane et al., 2001).

Sheep were chosen in this present study as an animal model to assess nasal absorption from the lyophilised nasal insert formulation. Nicotine hydrogen tartrate, known to be readily absorbed from the nasal mucosa in sheep (Cheng et al., 2002), was used as a model compound, and the lyophilised HPMC formulation was compared with nasally administered nicotine powder and solution formulations. This was designed to provide information on the effect of the lyophilised formulation on bioavailability, and a pharmacokinetic profile which may establish if any bioadhesive effect is present.

4.2 Materials and Methods

4.2.1 Chemicals

HPMC powder (grade K4MP) was obtained as a gift from Dow Chemicals (Michigan, USA). D (-) mannitol (GPR) was purchased from BDH (VRW, Poole, UK), and nicotine hydrogen tartrate (NHT) purchased from Sigma (Gillingham, UK).

4.2.2 Apparatus

Polythene microcentrifuge tubes (0.2mL) were obtained from Life Sciences (Basingstoke, UK). A Virtis Advantage Freeze Drier, which carried out a cycle with preset stages, was used to lyophilise HPMC gels (Virtis, NY, USA).

4.2.3 Methods

4.2.3.1 Preparation of HPMC gels

K4MP gel containing NHT was prepared as described in section 2.2.3.1, with the exception that NHT was added in an appropriate quantity to allow 4mg of nicotine base per insert.

4.2.3.2 Preparation of lyophilised inserts

HPMC gel containing 2% w/w K4MP, 1% w/w mannitol and NHT (4mg nicotine base/ 0.7mL) was lyophilised in 0.7mL microcentrifuge tubes as described in section 2.2.3.4. The insert was administered to the turbinate site in the sheep's nasal cavity by inserting the lyophilisate into a Portex Z79 naso-pharyngeal tube (internal diameter 7.0mm, external diameter 10.00mm, marked to ensure consistent 85mm depth of administration to the nasal cavity), and gently pushing it out using a flexible nylon rod. Figure 4.1 shows the positioning of delivery of a nasal insert to the turbinate site of the nasal cavity using the naso-pharyngeal tube.

4.2.3.3 Nicotine solution

NHT solution at a concentration of 4mg/200μL nicotine base was administered via silicone tubing attached to a 50mL syringe with puncture holes made in the tubing distal to the syringe. A Gilson pipette was used to measure 200μL of nicotine solution into the tubing which was inserted into the nasal cavity via the naso-pharyngeal tube, and the solution sprayed onto the turbinate site by depressing the syringe plunger. Figure 4.2 shows the assembled device for delivery of NHT solution to the nasal cavity, and Figure 4.3 shows the end of the silicone tubing with the puncture holes.

4.2.3.4 Nicotine powder

For each dose, NHT powder (~12mg) equivalent to 4mg of nicotine base was weighed out and packed into the last 2cm cut from the tip end of a 200μL Gilson pipette tip. An equal weight of mannitol powder was packed into the pipette tip, on top of the layer of NHT powder. For dosing, the narrow end of the pipette tip was inserted into a length of silicone tubing (Portex, Kent, UK, internal diameter 1.95mm, external diameter 5mm), with a 50mL syringe with the plunger fully withdrawn placed at the other end.

Depressing the plunger on the syringe created a rapid expulsion of air from the barrel, which propelled the powder from the pipette tip into the nasal cavity of the sheep. The powder was applied directly to the turbinate site of the nasal mucosa by inserting the pipette tip and tubing to the required depth into the nasal cavity via the naso-pharyngeal tube. Figure 4.3 shows the pipette tip and silicone tubing end of the device for administration of NHT powder to the nasal cavity.

4.2.3.5 Intra-venous

NHT solution was filter sterilised at a concentration of 0.2mg/mL nicotine base and 5mL of this solution (1mg nicotine base) administered by venepuncture into a jugular vein.

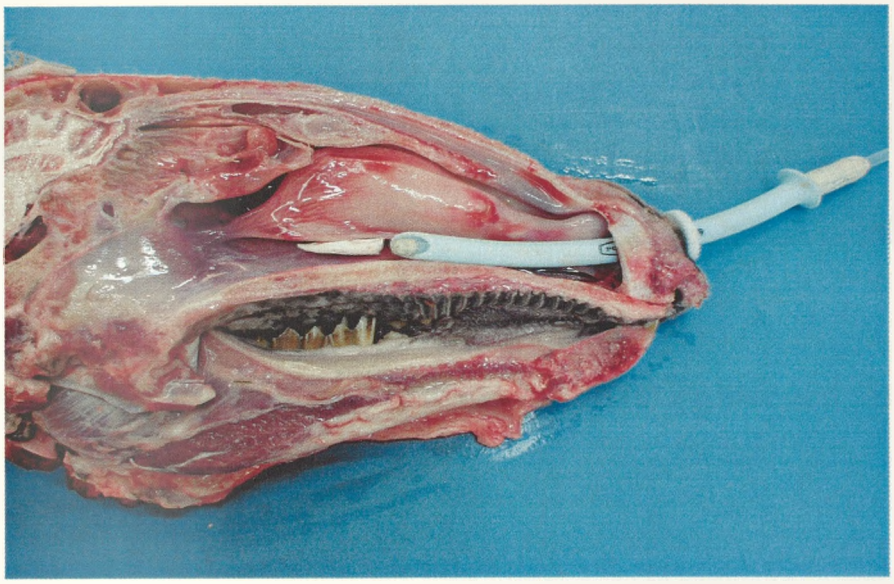


Figure 4.1 **Cross-section of sheep nasal cavity showing delivery of insert to the turbinate site.**

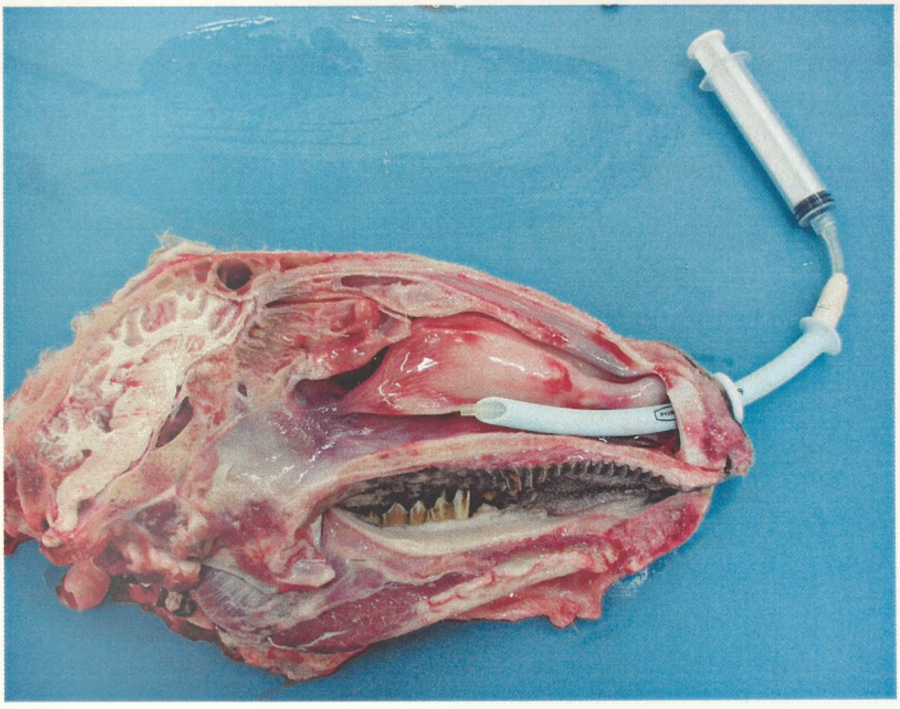
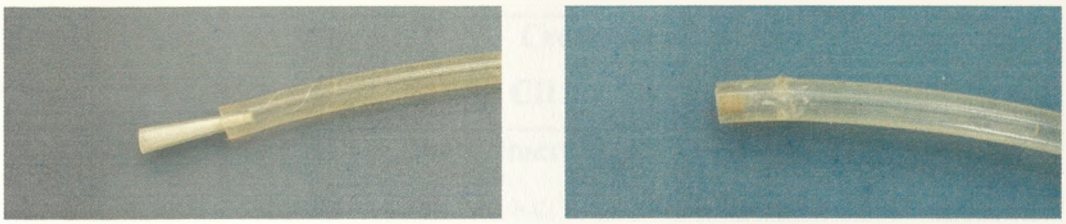


Figure 4.2 **Cross-section of sheep nasal cavity showing device for solution administration.**



(a) (b)

Figure 4.3 Administration device for delivery of (a) powder and (b) solution to nasal cavity.

4.2.3.6 Study protocol

Eight Wether sheep, 1-2 years old, weighing 50-70 Kg, were obtained from Cochno Farm and Research Centre (University of Glasgow, UK). They were housed indoors and received food and water *ad libitum* for the duration of the study, which was approved by the University of Glasgow Ethical Review Committee and performed under a Home Office (UK Government) Licence. A crossover study design was adopted (Table 4.1), with a wash out period of 7 days between treatments.

Table 4.1 Nasal absorption of NHT in sheep crossover study design.

Animal number	Crossover number			
	CI	CII	CIII	CIV
1	s.c.	Insert	Powder	Spray
2	Spray	s.c.	Insert	Powder
3	Powder	Spray	s.c.	Insert
4	Insert	Powder	Spray	s.c.
5	Spray	Powder	Insert	s.c.
6	Powder	Insert	s.c.	Spray
7	Insert	s.c.	Spray	Powder
8	s.c.	Spray	Powder	Insert

For serial blood sampling, an in-dwelling intravenous cannula (50mm x 16g, Dunwood, Aberdeen, UK) was placed in the jugular vein of each sheep, and retained in place for the duration of each experimental leg. The cannula was kept patent by flushing with heparinised normal saline when required, and was removed at the end of the blood sampling period. The sheep were not sedated to avoid impairing nasal mucociliary function (Mayor and Illum, 1997). A pre-dose blood sample was taken prior to NHT administration, following which blood was sampled at 15, 30, 45, 60, 90, 120, 180, 240, 360 and 480 minutes after dosing, except for IV administration when sampling was at 2, 5, 10, 15, 20, 30, 45, 60, 90, 120, 180, 240, 360 and 480 minutes. Blood samples were collected in heparinised Vacuette tubes (Lithium Heparin 9mL Monovette, Sarstedt, Leicester, UK), plasma was separated by centrifugation at 3000 x g for 15 minutes and stored at -20°C until analysis.

Administration of doses and blood sampling was carried out by Home Office personal licence holders of the University of Glasgow. With assistance from staff of the Department of Veterinary Pharmacology, University of Glasgow, the author was responsible for overseeing the study, ensuring that dosing and sampling were carried out at the appropriate time points, and that the samples obtained were correctly

labelled. The author also undertook plasma separation of at least half of the blood samples obtained, and assisted in general handling of the sheep.

4.2.3.7 Analysis of plasma samples

Plasma samples (1mL) spiked with 50ng of internal standard (nicotine-methyl-d_3) and mixed with 0.5mL of 0.1M sodium phosphate buffer (pH 3) were filtered using Whatman 0.45μm 22mm Nylon GD/X syringe filters. The filtrate was extracted on an Isolute^R SCX column, the analyte eluted with 1mL of 3% w/v in ammonia in methanol solution and then blown to near dryness under a stream of nitrogen (Figure 4.4). The remaining concentrated solution was transferred to sample vials for LC-MS analysis on an Automass GC/LC-MS system. Selected ion monitoring was carried out for the base peak of the electron mass spectra of nicotine (m/z 84) and nicotine-methyl-d_3 (m/z 87) (Figure 4.5).

The analysis was carried out with the assistance of the Pharmaceutical Analysis Department of the University of Strathclyde. The author analysed the samples of four sheep from one crossover of the study.

4.2.3.8 Pharmacokinetic data analysis

The data were analysed assuming first-order absorption and one compartment model kinetics with first order elimination. Maximum plasma concentration (C_{max}) and time of its occurrence (T_{max}) were calculated directly from the plasma concentration against time data for all four NHT formulations. The area under the curve from 0 to 8 hours was calculated using the trapezoidal rule.

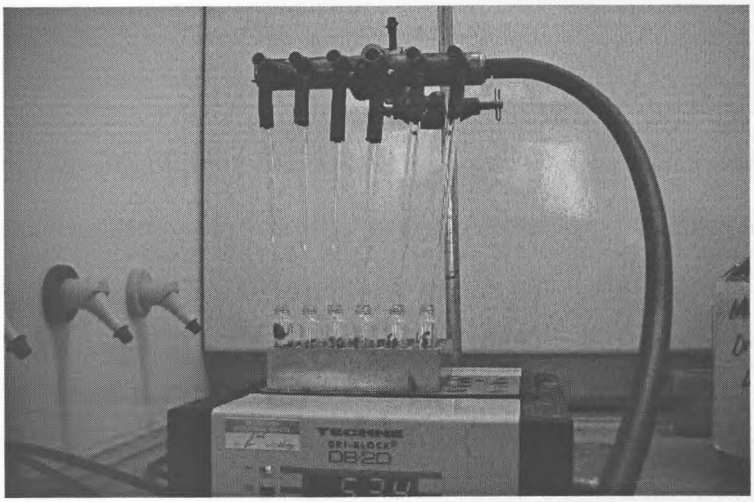


Figure 4.4 Samples drying under a nitrogen stream.

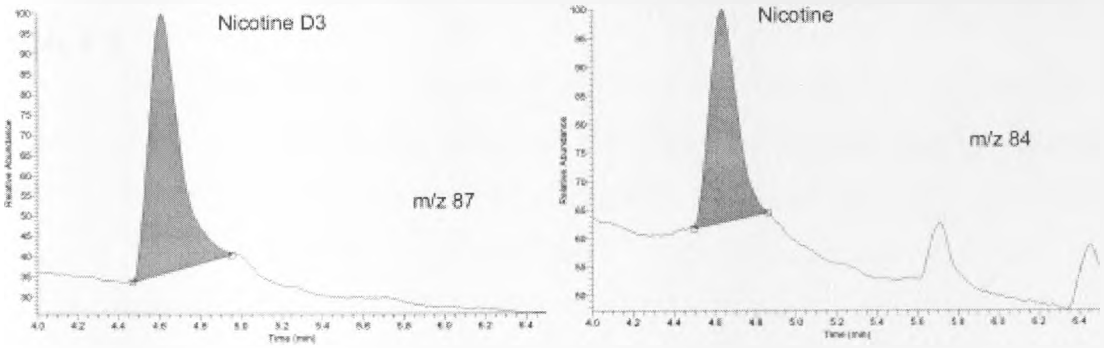


Figure 4.5 Nicotine selected ion monitoring.

4.3 Results and Discussion

4.3.1 *In-vivo* nicotine plasma concentrations and pharmacokinetic analysis

The plasma nicotine concentrations as determined using LC/MS are shown for all four formulations in Appendix A.

The limit of quantitation for nicotine in plasma in the LC/MS analysis was considered to be 4ng/mL (values shown in italics in Appendix A). Therefore, following the time point where plasma nicotine fell below this level, subsequent values were considered to be invalid and excluded from calculations. The IV data was then normalised to a 4mg dose, to allow comparison among formulations. Other data excluded from calculations were the 15 minute nasal spray data points for sheep 2 and 4 which were considered to be anomalous, being much higher than would be expected given the plasma profiles of the other sheep, and subsequent data points for these two sheep, and the 90 minute nasal spray data point for sheep 2, which was considered anomalously low given the surrounding data points of the plasma profile. The pharmacokinetic data analysis (incorporating the listed exclusions) is shown in table 4.2.

Table 4.2 Pharmacokinetic parameters (mean values ± s.d.) following intranasal and intravenous administration of nicotine in sheep (IV data normalised to a 4mg dose).

Formulation	T_{max} (min)	C_{max} (ng/ml)	AUC (ng.h/ml) (0 – 480 minutes)
IV normalised (n = 8)	2.80 ±1.4	37.7 ±23	65.2 ±129
Lyophilised Insert (n = 7)	39.4 ±33	27.6 ±23	54.4 ±69
Spray (n = 6)	21.0 ±13	45.6 ±24	33.6 ±33
Powder (n = 6)	20.0 ±7.7	36.4 ±19	17.7 ±6.4

The plasma concentration vs time profiles for the four formulations in sheep (with the IV data normalised to a 4mg dose) indicated that the nasal powder and liquid doses showed a similar profile to that of the IV dose after C_{max} had been reached, plasma levels tailing off quite rapidly (Figure 4.6). Thirty minutes after dosing, plasma nicotine concentrations for nasal NHT powder and solution were approximately one half and one quarter of their peak values respectively, and at 90 minutes the amount of nicotine detected in the plasma was negligible. Absorption profiles for the nasal powder and solution preparations, which are very similar to that of the IV dose, with a rapid increase to the peak nicotine absorption, followed by a very similar sharp decrease in plasma levels, are consistent with the results of Jung et al (Jung et al., 2000).

The lyophilised nasal insert, on the other hand, gave a different plasma profile. An initial plasma peak (lower than for the other formulations) was followed by a fall in plasma nicotine concentration, then an extended absorption phase so that significant plasma nicotine levels were sustained over approximately 2 hours. This

was followed by a gradual fall in plasma levels, at a much slower rate than for the other nasal preparations and the IV injection.

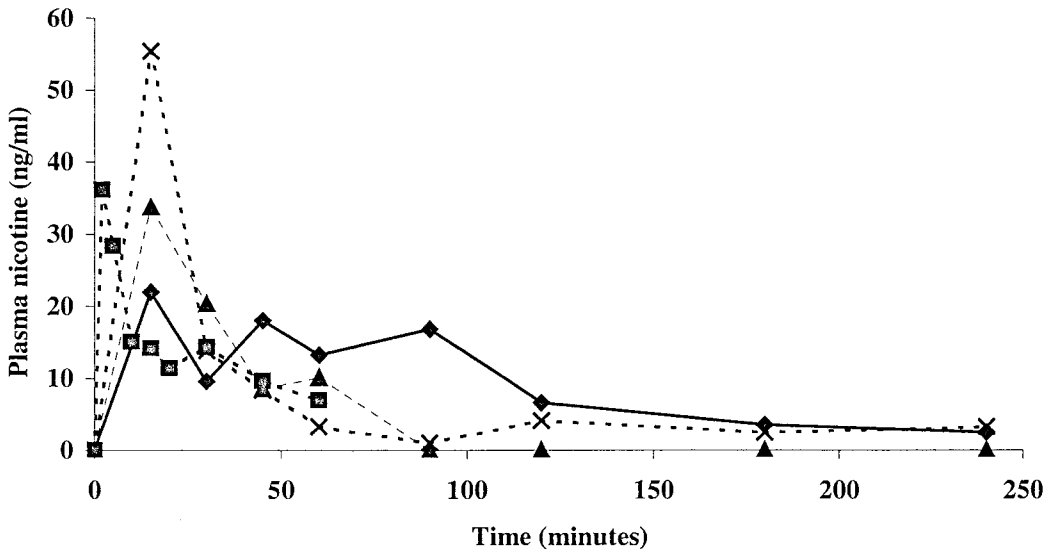


Figure 4.6 **Mean plasma concentration time profiles (displayed to 240 minutes for clarity) of different formulations following intranasal and intravenous administration of 4 mg of nicotine in sheep.**
(■) IV (◆) nasal insert (x) nasal spray (▲) nasal powder.

The IV dose gave rise to a rapid T_{max} of 2.80 ±1.4 minutes and the nasal powder and spray formulations T_{max} values were 20.0 ±7.7 and 21.0 ±13 minutes respectively. The nasal insert, on the other hand, yielded a mean T_{max} of 39.4 ±33 minutes, demonstrating delayed release and absorption of nicotine from this formulation. Corresponding C_{max} values decrease in the order nasal spray > IV > nasal powder > nasal insert. The mean AUC of the insert was markedly greater than that for the other nasal formulations, and approximately 80% that of the normalised IV dose. However, there was significant variation amongst animals, and consequently statistical analysis was not carried out.

The T_{max} values could be ranked lyophilised insert > nasal spray > nasal powder > IV (Table 4.2), indicating delayed absorption from the nasal tract which prolonged the time to peak plasma nicotine level. As far as the lyophilised insert was concerned the long T_{max} is likely to result from a combination of two factors. Firstly, the insert would require time for hydration of the polymer on the nasal mucosa before nicotine release would begin, a factor not involved in absorption from the nasal solution or readily soluble powder formulations from which the NHT should be immediately available. This is reflected in the relatively rapid plasma nicotine peaks (around 20 minutes) for these formulations, and the T_{max} value of the nasal insert which is far greater than the IV dose, and also higher than those from the powder and spray doses.

The second, probably more significant, contribution to the delayed absorption from the nasal insert would be the viscous nature of the hydrated gel layer, through which the nicotine has to diffuse to be released. This is consistent with the *in-vitro* results, and can account for the prolonged second absorptive phase observed in the plasma profile of the lyophilised insert. By adhering to the mucosal surface the hydrated polymer matrix would make drug available for absorption over an extended period.

A combination of these two factors may explain the apparent "double peak" of the plasma profile for the lyophilised insert. The preliminary plasma nicotine peak could be attributed to an initial release of nicotine from the surface of the insert, with the secondary peak being a result of absorption following diffusion of nicotine through the slowly hydrating formulation, assumed to be slowly spreading over the surface of the nasal mucosa. This effect is similar to plasma profiles reported by Cheng et al., 2002, who used an ion exchange amberlite resin to achieve pulsatile and sustained plasma nicotine profiles in sheep. The lyophilised insert formulation may therefore have potential applications for any drug for which a sustained level of drug absorption is required, for example nicotine as a smoking cessation aid, providing an initial burst followed by a sustained supply of nicotine (in a similar manner to transdermal patches currently available).

A further mechanism of action on the nasal mucosa has been suggested for bioadhesive formulations, that of water uptake affording a transient opening of the

tight junctions between the cells of the nasal mucosa, allowing an alternative path of transport of drug than through aqueous pores. This opening of the tight cell junctions is suggested to be caused by dehydration of mucosal cells as a result of water uptake by the bioadhesive formulation (Edman et al., 1992). If this effect does occur, then the insert formulation is likely to trigger this effect, due to its requirement for hydration by water from the nasal mucosa, and the demonstrated uptake of water and swelling of HPMC (Columbo et al., 1999). However, this route of absorption may not be of importance for a compound such as nicotine, which as the base compound would be readily absorbed across the mucosa.

The extended absorptive phase of the nasal insert suggests prolonged nasal residence of the formulation. Continued absorption of nicotine suggests that the lyophilised insert remains in the nasal cavity releasing nicotine for approximately 2 – 3 hours. *In-vitro* observations show that a viscous bioadhesive gel is formed on hydration of the dosage form. If this gel is formed on administration of the insert to the sheep nasal cavity, then it would be expected to resist the rapid mucociliary clearance rate by adhering to the mucosa.

Comparison of the AUC values for the three nasal preparations demonstrate that the lyophilised insert formulation gave greater systemic bioavailability of nicotine than either the nasal powder or solution formulations. Bioavailability of the four formulations could be ranked in the order IV > lyophilised insert > nasal spray > nasal powder. The lyophilised insert displayed an encouraging bioavailability, just less than that of the IV formulation and greater than both the nasal powder and solution formulations, demonstrating that despite the lower C_{max} the extended absorptive profile of the nasal insert enhanced overall NHT absorption in comparison with the conventional nasal formulations.

Differences in metabolic rate may in part explain the variability among the data obtained from the different animals. It is also possible that on administration of some of the nasal formulations, the nasal mucosa of the sheep may have been temporarily compromised in some way, resulting in higher than anticipated plasma levels. Alternatively, if the nasal dose were not delivered effectively to the correct site on the nasal mucosa for some reason, the plasma levels obtained for the

formulation in that particular sheep may have been lower than those observed for the same formulation in the other sheep.

4.4 Conclusion

The data obtained demonstrates that a lyophilised nasal insert formulation provides an entirely different nicotine absorption profile than those obtained with conventional nasal formulations and an IV dose. The insert displayed a delayed T_{max}, with a prolonged absorptive phase in comparison with the other formulations. The results suggest that the insert formulation yields increased bioavailability over the conventional nasal powder, liquid or spray preparations, and that the bioadhesive nature of the formulation confers prolonged opportunity for nasal absorption. These advantages demonstrated by the insert formulation show potential for the application of the dosage form to improve absorption of drugs which suffer from a low nasal bioavailability, without the use of absorption enhancers.

Chapter 5 *In-vivo* Evaluation of Lyophilised Insulin Insert in Sheep

5.1 Introduction

When delivered orally insulin is absorbed only to a very small extent as a result of chemical and enzymatic degradation in the GI tract, the peptides' large molecular weight and hepatic first pass metabolism, and as a result subcutaneous (s.c.) administration of insulin is carried out by insulin dependant diabetic patients on a daily basis to maintain control of blood glucose levels. This can often be an uncomfortable or painful process, and as a result, achieving adequate nasal absorption of insulin as an alternative to s.c. dosing has been the focus of many researchers in this area.

Animal models have been used to examine the extent of nasal absorption of various peptides, such as LHRH in sheep (Illum et al., 2000), G-CSF in sheep (Gill et al., 1998), calcitonin in rats (Morimoto et al., 2001, Morimoto et al., 1985), and desmopressin in rats and sheep (Critchley et al., 1994), all with the aim of by-passing the first pass metabolism effect. *In-vivo* investigation of nasal insulin absorption was first reported in dogs (Hirai et al., 1978), and is frequently reported in association with the use of absorption enhancers in attempts to improve the low nasal bioavailability of the high molecular weight hormone. A summary of some of the effects of some absorption enhancers used is shown in Table 5.1.

Table 5.1 Agents used to enhance nasal absorption of insulin in animals.

Species	Insulin dose	Absorption enhancer	Insulin C_{max} (μiu/mL)	Bioavailability as a % relative to (X)	Reference
Dog	50 iu	Sodium glycocholate or polyoxyethylene-9-lauryl ether	75%*	-	Hirai et al., 1978
Sheep	100 iu	Chitosan/ Chitosan nanoparticles	743 ±259/ 106 ±99	17.0 ±7 (s.c.)/ 1.3 ±0.8 (s.c.)	Dyer et al., 2002
Sheep	2 iu/Kg	Lysophosphatidyl choline Glycodeoxyxholate Sodium taurodihydrofusidate	380 ±58 776 ±155 409 ±59	25.3 (s.c.) 31.9 (s.c.) 16.5 (s.c.)	Illum et al., 2001a
Sheep	1.39 iu/Kg	Sodium taurodihydrofusidate	1250	37.8 ±8.4 (IV)	Lee et al., 1991
Rabbit	2/4 iu	Dimethyl-β-cyclodextrin	640 ±104	2.8 ±3 12.9 ±4.4 (a)	Schipper et al., 1993
Rabbit	28 iu	Sodium polystyrene sulphonate	413 ±72	6.5 (i.m.)	Takenaga et al., 1998
Rabbit	15.8 iu	Glycofurol	41%*	-	Bechgaard et al., 1996
Rabbit	10 iu	Maltodextrin	3668 ±82	8.7 ±2.6 (IV)	Callens and Remon, 2000
Sheep	1 iu/Kg	Sodium taurodihydrofusidate	-	16.4 ±2.4 (IV)	Longenecker et al., 1987

*decrease in blood glucose.
(a) % of dose received.

Other researchers have studied bioadhesive materials to increase nasal residence of insulin in attempts to improve bioavailability. Chitosan, a bioadhesive cationic polymer thought to also cause a transient opening of tight cell junctions has been used to enhance absorption of insulin in rats (Aspden et al., 1996), rabbits (Fernandez-Urrusuno et al., 1999) and sheep (Illum et al., 1994a, Dyer et al., 2002). Bioadhesive starch microspheres have been shown to produce a nasal insulin bioavailability of 10.7% (±2.6) in sheep (Farraj et al., 1990), an effect which is increased to between 6.4–31.5% on addition of various combinations and concentrations of absorption enhancers (Farraj et al., 1990, Illum et al., 2001a). The authors found a synergistic action, and suggested this was due to a combination of the bioadhesive nature of the microspheres increasing contact time for insulin absorption and opening of tight junctions, and the differing actions of the enhancers.

The mechanism of the insulin nasal absorption enhancing effect of starch microspheres was first shown by Edman et al., 1992 in rabbits, where a transient, reversible opening of mucosal cell tight junctions was observed, which the authors suggested to be due to the water holding capacity of the microspheres dehydrating and shrinking the cells. This concept was further investigated with the addition of the osmotic agents glycerol and creatinine to ethyl (hydroxyethyl) cellulose gel, which resulted in further lowering of blood glucose in rats compare to the gel alone (Pereswetoff-Morath and Edman, 1995). The authors of Farraj et al., 1990 further investigated the effect by demonstrating that hyaluronic acid microspheres produced similar plasma insulin levels in sheep (Illum et al., 1994b) as they had previously observed with starch microspheres.

Bioadhesive formulations which are capable of swelling may then offer a suitable combination of properties (Ryden and Edman, 1992) for promotion of nasal insulin absorption without the use of potentially harmful absorption enhancers.

In common with many proteins, insulin is a fragile molecule, sensitive to the chemical environment in which it is formulated, and various physical influences such as storage temperature and the nature of the storage container used. It is known that protein structure can be highly sensitive to the effects of pH, as the charge present on the sixteen ionisable amino acid groups contained within the molecule will greatly affect the electrostatic interactions which contribute to the tertiary structure of the

protein, essential for biological activity. The biologically active native insulin is composed of two amino acid chains (A and B) connected by two disulphide bonds, with an inter-chain disulphide bond on the A chain (Figure 5.1)

The formation of biologically inactive insulin aggregates is a common problem in insulin solutions, causing instability and precipitation, often with subsequent blockage equipment such as insulin infusion pumps, and underdosing due to lack of pharmacological activity of the aggregates (Sefton and Antonacci, 1984, Lougheed et al., 1980). One piece of research demonstrated the effect of the container used to store insulin solutions, by measuring adsorption isotherms of various materials (Sefton and Antonacci, 1984), and found that hydrophobic materials such as Teflon or silicon rubber induced more adsorption of insulin to the surface than hydrophilic material such as glass or poly acrylamide. It was also found that incubation time increased adsorption to the surface of the material, and that decreasing pH to 3.5 from 7.4 also increased adsorption.

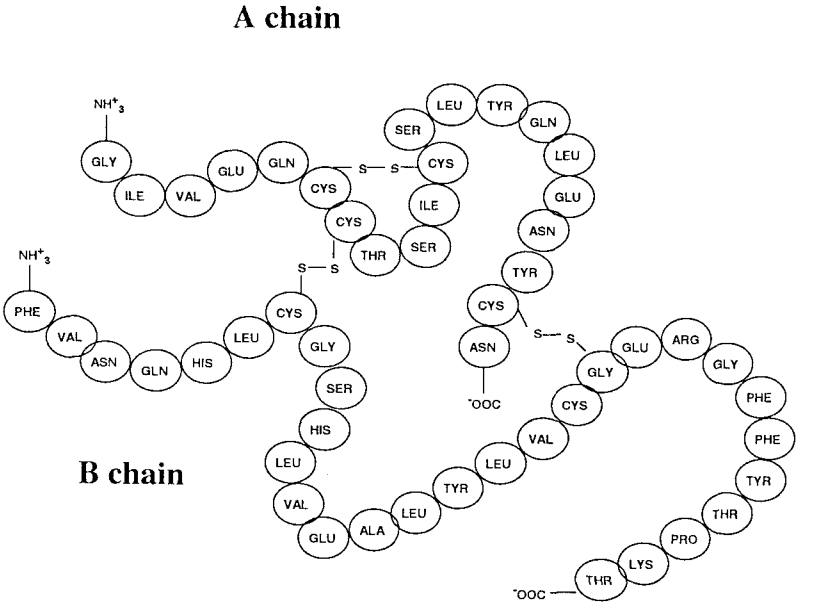


Figure 5.1 **Primary structure of insulin.**

Redrawn from Costantino et al., 1997.

The process of adsorption to a hydrophobic surface is thought to be a first step in the formation of aggregates, whereby a conformational change exposes hydrophobic regions in the molecule, allowing aggregation to occur via non-covalent forces (Sluzky et al., 1992) (Figure 5.2).

This study demonstrated that aggregation of insulin in solution was far greater within containers manufactured from hydrophobic material than glass, confirming that hydrophobic materials play a role in aggregation.

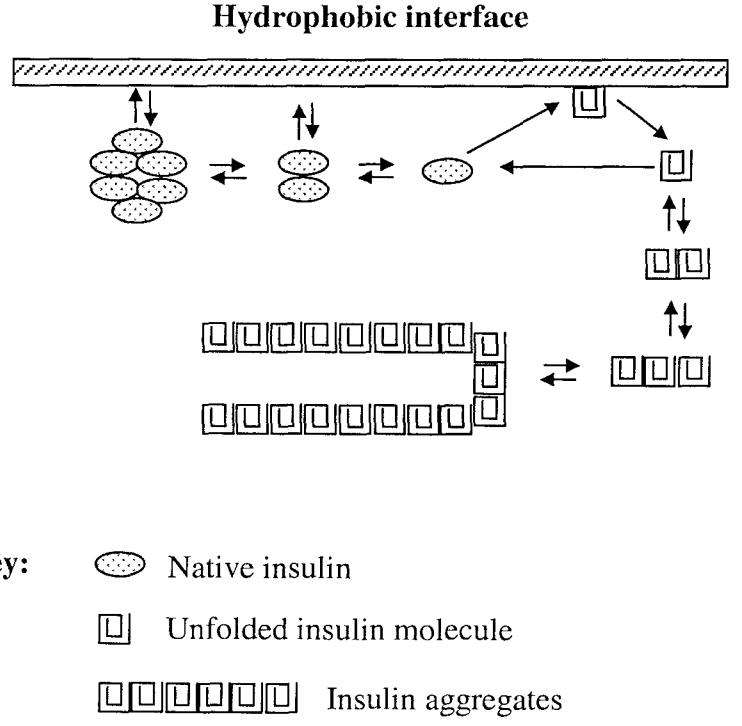


Figure 5.2 **Mechanism of insulin aggregation at a hydrophobic interface.** Redrawn from Sluzky et al., 1992.

The air:water interface in an insulin solution has also been identified as a hydrophobic surface responsible for aggregation (Sluzky et al., 1992, Lougheed et al., 1983, Dathe et al., 1990), and these studies have shown that agitation of the solutions induces aggregation. Insulin solution concentration also influences aggregation, with increased insulin concentration generally increasing the tendency to aggregate (Costantino et al., 1997, Treuheit et al., 2002), as the opportunity for molecular interaction increases.

The pH of the solution also plays a role in this process, as lower pH favours native insulin, the species involved in aggregation, resulting in increased aggregation potential. However, a reversal of this effect was found in a study where agitation was introduced into the system, which was expected to further increase the aggregation of solutions of high protein concentration (Treuheit et al., 2002), by increasing the area of the air: water interface. The opposite was found to be true, with the researchers proposing that the critical factor in an agitated system was the air:water interface to protein concentration ratio, meaning that as this ratio decreases (with increasing peptide concentration), so the tendency to aggregate decreases. In non-agitated systems, it is suggested, the rate limiting mechanism for aggregation is the collision of protein molecules in solution, accounting for the increase in aggregation upon storage as protein concentration increases.

Research has shown that the introduction of surfactants such as polysorbate (Treuheit et al., 2002), and polypropylene glycol/polyethylene glycol block polymers (Thurow and Geisen, 1984), can stabilise protein solutions against aggregation at hydrophobic surfaces. It was suggested that the presence of surfactant at the air:water interface removes this surface as a potential aid to aggregation (Treuheit et al., 2002), and that in the case of the block polymer, hydrophobic regions on the polymer bind to the interface, leaving hydrophilic regions protruding into the solution, imparting hydrophilic qualities to the surface (Thurow and Geisen, 1984). Other mechanisms known to be involved in insulin aggregation include the presence of moisture in lyophilised powders, increase in temperature, pH (Costantino et al., 1994, Lougheed et al., 1980), and the presence of zinc (increasing the tendency to form hexamers) (Milthorpe et al., 1977, Jeffrey, 1974).

In solution insulin exists in equilibrium with native insulin, dimers and hexamers of insulin. The pH of the solution can greatly affect this equilibrium by altering the ionisation state of amino acid groups, which in turn affect the association state of the insulin molecules. It has been stated that neutral pH favours the stable hexamer in solution, while acidic pH favours the native species which is involved in aggregation (Costantino et al., 1997). While this has obvious implications for stability, the insulin hexamer is biologically inactive, and in any case such a large molecular weight aggregate is even less likely to be able to cross the nasal mucosa than the native insulin species. At pH 4.5-6.5, insulin is insoluble as a result of isoelectric precipitation, due to its isoelectric point (p*I*) being 5.5. It is known that below pH 4.5, formation of the insulin hexamer is disrupted due to protonation of the B_{10} histidine amino acid moiety (Bryant et al., 1993), and so the effects of acidic pH on insulin solution were investigated. It was reported that acidic pH (studied as low as pH 2) gave conformational stability to insulin as a result of the protonation of the B_5 histidine group, which has a pKa of 7.0, in a similar manner to the acidic environment in which it is stored in secretory granules in the body. However, the protonated B_5 histidine group may decrease the conformational flexibility of insulin, necessary for receptor binding and bioactivity. The researchers suggested that protonation of this group may act as a functional 'switch' between storage stability and bioactivity (Bryant et al., 1993).

It is clear that many formulation factors can influence the behaviour of insulin, and that in many cases, it may be necessary to obtain a balance between the stability of the formulation, and the activity and availability for nasal absorption. Therefore an initial investigation of nasal insulin absorption in sheep was followed by a study of the effect of formulation pH on nasal insulin absorption.

5.2 Materials and Methods

5.2.1 Chemicals

HPMC powder (grade K4MP) was obtained as a gift from Dow Chemicals (Michigan, USA). D (-) mannitol (GPR) was purchased from BDH (VRW, Poole, UK), and human recombinant insulin (expressed in Ecoli) was purchased from Sigma (Gillingham, UK). Commercially available, rapid acting, insulin solution (Humulin S) for subcutaneous injection was purchased from Lilley (Indianapolis, USA).

5.2.2 Apparatus

Polythene microcentrifuge tubes (0.6mL) were obtained from Life Sciences (Basingstoke, UK). A Virtis Advantage Freeze Drier, which carried out a cycle with preset stages, was used to lyophilise HPMC gels (Virtis, NY, USA).

5.2.3 Methods

5.2.3.1 Preparation of HPMC gel

K4MP gel containing insulin was prepared as described in section 2.2.3.1, with the exception that insulin was added in an appropriate quantity to allow 110iu of insulin per insert.

5.2.3.2 Preparation of lyophilised inserts

Lyophilised inserts were prepared and administered to the sheep as described in section 4.2.3.2, with the exception that the inserts contained 110iu insulin in place of NHT.

5.2.3.3 Insulin solution

Insulin solution was prepared by dissolving an appropriate amount of insulin in 0.01M HCl, adding dropwise amounts of NaOH to adjust the pH to pH 7.4, and adjusting the final volume to a concentration of 110iu/200μL. The solution was administered as described in section 4.2.3.3.

5.2.3.4 Insulin powder

Insulin powder was prepared and administered as described in section 4.2.3.4, with the exception that each dose contained 110iu insulin (~3.85mg) in place of NHT, and the weight of mannitol powder added was 10mg, rather than a weight equal to that of the insulin powder.

5.2.3.5 Subcutaneous

Commercially available insulin solution (100iu/mL) was injected subcutaneously to give a dose of 11iu (0.11mL). Also, a lyophilised 2% K4MP insert was reconstituted and diluted by a factor of 10 with water to give a dose of 11iu and administered subcutaneously in order to determine the level of activity of insulin in the formulation.

5.2.3.6 Study protocol

5.2.3.6.1 Pilot investigation

Eight wether sheep, 1-2 years old, weighing 50-70 Kg, were obtained from Cochno Farm and Research Centre (University of Glasgow, UK). They were housed indoors and received food and water *ad libitum* for the duration of the study, which was approved by the University of Glasgow Ethical Review Committee and performed under a Home Office (UK Government) Licence. A single dose, four sheep pilot study was carried out to investigate the feasibility and appropriateness of performing a full-scale investigation of nasal absorption of insulin in sheep. Each sheep received one of the four insulin doses described above. Serial blood sampling was carried out by venepuncture at –5, 2, 5, 10, 15, 20, 30, 45, 60, 90, 120, 180, 240, 300 and 360 minutes and samples were collected in 7.5mL potassium EDTA tubes (S-monovette, Sarstedt, Leicester, UK), as heparin was incompatible with the insulin assay. Collection of samples by venepuncture rather than via an in-dwelling catheter eliminated the requirement to flush with heparin, further avoiding assay contamination. Blood glucose levels were determined at the time of sampling using a handheld PocketScan glucometer (Lifescan, Loudwater, UK), following which plasma was separated by centrifugation at 3000 x g for 15 minutes and stored at -20°C until analysis. The sheep were not sedated to avoid impairing the mucociliary function.

Administration of doses and blood sampling was carried out by Home Office personal licence holders of the University of Glasgow. With assistance from staff from the Department of Veterinary Pharmacology, University of Glasgow, the author

was responsible for writing amendments to the Home Office Project Licence (including background information on the safety and relevance of intranasal insulin administration), overseeing the study, ensuring that dosing and sampling were carried out at the appropriate time points, and that the samples obtained were correctly labelled. The author also undertook plasma separation of at least half of the blood samples obtained, and assisted in general handling of the sheep.

5.2.3.6.2 Four way crossover

The study was carried out under the protocol described in section 5.2.3.6.1, with the exception that eight sheep were used in a four way cross-over study design, with a wash out period of 7 days between treatments, and that blood samples were taken at –15, -10, -5, 5, 10, 15, 20, 30, 45, 60, 90, 120, 150, 180, 210, 240, 300, 360, 420 and 480 minutes. This sampling regime allowed for establishment of baseline glucose and insulin values, and a more accurate assessment of the duration of effect of the doses.

5.2.3.6.3 Effect of formulation variables

The study was carried out under the protocol described in section 5.2.3.6.2, with the exception that eight sheep were used in a three way cross-over study design, with all eight sheep receiving a 2% K4MP insulin nasal insert formulated at pH 3, a 1% K4MP nasal insert formulated at pH 7, four sheep receiving a placebo 2% K4MP nasal insert, and four sheep receiving the reconstituted s.c. dose. Formulating the nasal insert dose at pH 3 was considered to be suitable as the findings of Pujara et al., 1995 showed that intracellular protein release from rat nasal mucosa (used as a measure of mucosal cell breakdown) was similar in the range pH 3-10. The 1% K4MP formulation was included to examine the possibility that the increased insulin release of this formulation demonstrated *in-vitro* in comparison with the 2% K4MP insert in Chapter 3 may improve nasal insulin absorption.

5.2.3.7 Stability of blood glucose readings

Blood glucose stability was checked in order to assess if a slight delay in the time taken to perform the reading due to unforeseen circumstances would affect the results obtained. Random blood samples were selected during the study and a blood glucose reading was taken at 0, 5, 10, 15, 20, 30, 45, 60, 90, 120 minutes after sampling.

The author carried out glucose analysis of one quarter of the samples produced, with assistance from students of the Department of Pharmaceutical Sciences, University of Strathclyde.

5.2.3.8 Analysis of plasma samples

When required for analysis, samples were thawed to room temperature, and the insulin content determined using an ICN Biomedicals Iodine^{125} radioimmunoassay (ICN Biomedicals, Basingstoke, UK).

Plasma (0.1mL) was added to antibody-coated tubes, vortexed with 0.9mL I^{125} buffer and the tubes were incubated for 18 hours, along with calibration standards, which had been prepared in the same manner. The tubes were then aspirated and washed twice with distilled water. Remaining radioactivity was counted using a gamma counter, and plasma insulin levels determined from the standard curve prepared. Insulin competitively binds with the antibody contained within the tubes, meaning that the higher the quantity of insulin in the sample, the less I^{125} will bind with the antibody, giving a lower gamma activity count.

The author undertook analysis of half of all samples, with the assistance of staff at the Department of Veterinary Pharmacology, University of Glasgow.

5.2.3.9 Pharmacokinetic data analysis

The data were analysed assuming first-order absorption and one compartment model kinetics with first order elimination. Maximum plasma insulin concentration (C_{max}) and time of its occurrence (T_{max}) were calculated directly from the plasma concentration vs time data the insulin formulations. The area under the curve from 0 to 6 hours for the pilot study, or 0 to 8 hours for the crossover study was calculated using the trapezoidal rule, and the bioavailability of the intranasal (IN) formulations relative to the s.c. dose (F_{rel}) was calculated using an equation similar to that described by (Lee et al., 1991):

$$F_{rel} = \frac{(AUC_{IN} / Dose_{IN})}{(AUC_{s.c.} / Dose_{s.c.})} \times 100$$

This equation was used to allow a direct comparison between the formulations, accounting for the difference in dose between the s.c. and nasal preparations, as the normalisation of data as carried out in Chapter 4 for IV NHT was not considered valid for a pharmacological response such as lowering of blood glucose.

Blood glucose as a percentage of the baseline value was calculated using an equation modified from that described by (Hirai et al., 1981a):

$$\text{Decrease (\%)} = \frac{Glucose_{(baseline)} - Glucose_{(sample)}}{Glucose_{(baseline)}} \times 100$$

5.3 Results and Discussion

5.3.1 Stability of blood glucose readings

All blood samples tested (n=3) showed good stability of blood glucose readings over a period of two hours, with maximum variation being ±0.4 mmol/L.

5.3.2 *In-vivo* blood glucose and plasma insulin concentrations and pharmacokinetic analysis

5.3.2.1 Pilot investigation

The blood glucose profiles obtained for each formulation in a single sheep were calculated as a percentage of the baseline value obtained at –5 minutes, and are shown in Figure 5.3. Plasma insulin levels were calculated as an increase from the baseline –5 minute value (Figure 5.4).

Blood glucose levels for the nasal powder and nasal spray showed little change during the time period studied. Both formulations gave a slight (11% and 6% below baseline) decrease in blood glucose 15 minutes after dosing, following which the nasal spray values returned to baseline values for the remainder of the study period, the nasal powder displaying similar behaviour until 180 minutes where a decrease to 80% of baseline plasma glucose is observed. This is unlikely to be due to the administration of the nasal insulin powder, as it would be expected to have been cleared from the nasal cavity by this time. A high glucose reading at 15 minutes of 175% increase from baseline for the nasal insert is thought to be a false reading.

The s.c. dose gave a decrease to approximately 50% of baseline values, and the nasal insert caused a similar, although slightly delayed decrease in blood glucose. This delay in effect is similar to the findings for nasal NHT absorption discussed in Chapter 4, and is consistent with the theory that the time required for hydration of the lyophilisate and release of drug through the polymer matrix will result in a delayed onset of action.

Plasma insulin profiles reflected the results obtained for blood glucose measurements, except for the nasal spray 60 minute insulin value which was uncharacteristically high. This point was considered to have been a result of an analysis error, and Figure 5.5shows the insulin concentration time profile when this data point has been omitted, to enhance clarity of the other profiles.

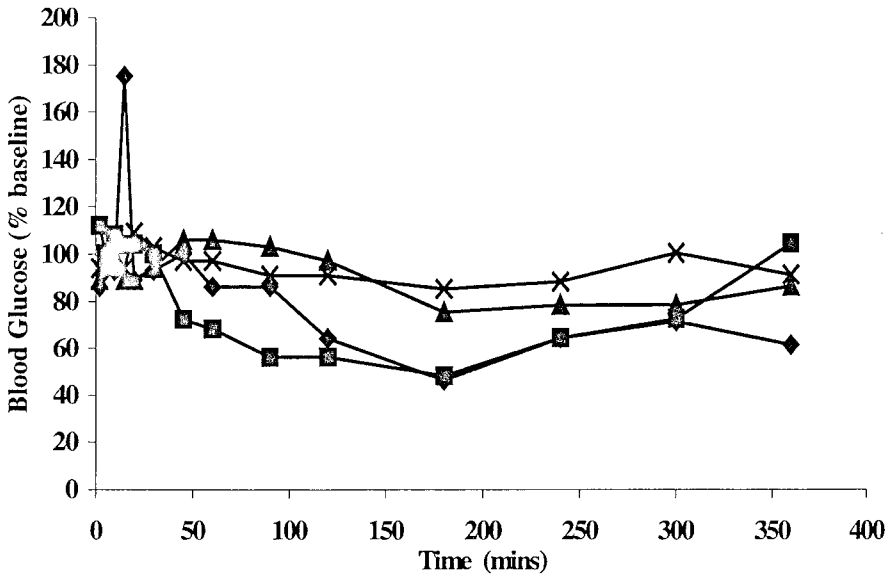


Figure 5.3 **Blood glucose concentration time profiles (expressed as a percentage of the baseline value) of different formulations following intranasal (110iu) and s.c. (11iu) administration of insulin in sheep (n=1)**

(■) s.c. (◆) nasal insert (X) nasal spray (▲) nasal powder.

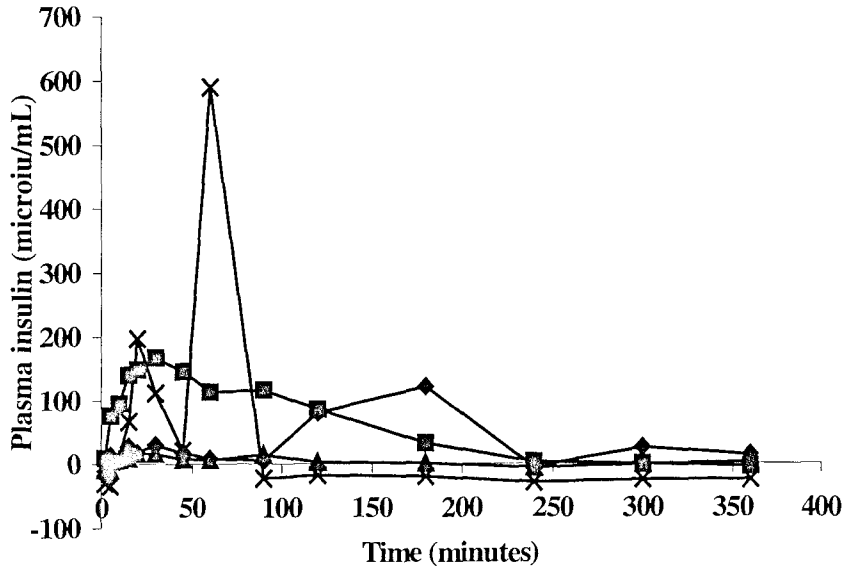


Figure 5.4 **Plasma insulin concentration time profiles (expressed as the increase from the baseline value) of different formulations following intranasal (110iu) and s.c. (11iu) administration of insulin in sheep (n=1).**

(■) s.c. (◆) nasal insert (x) nasal spray (▲) nasal powder.

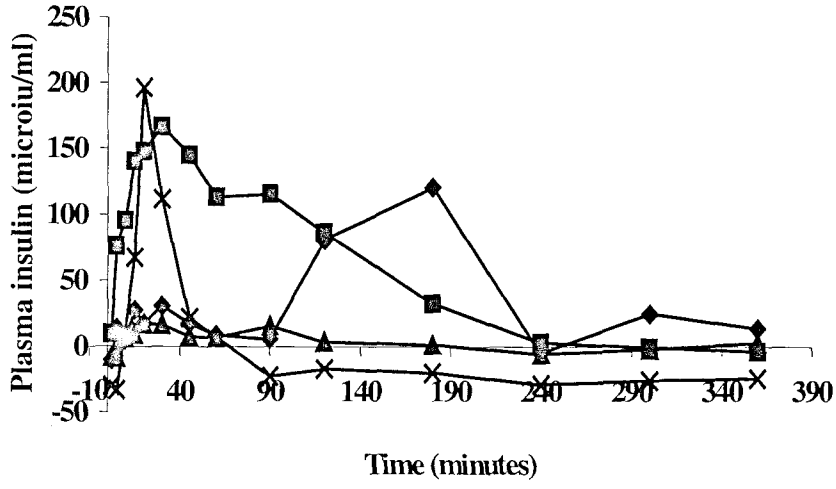


Figure 5.5 **Plasma insulin concentration time profiles (expressed as the increase from the baseline value, shown without anomalous data point for clarity) of different formulations following intranasal (110iu) and s.c. (11iu) administration of insulin in sheep (n=1).**

(■) s.c. (◆) nasal insert (x) nasal spray (▲) nasal powder.

Nasal insulin powder did not result in a rise in plasma insulin, which is consistent with blood glucose levels, while the nasal spray resulted in a rapid peak in blood insulin which was higher than that observed for the s.c. dose, although it was noted that the corresponding decrease in blood glucose was minimal. The s.c. dose gave rise to a reasonably rapid increase in plasma insulin levels as expected, and the nasal insert produced an increase of a similar magnitude, with a delay in onset as observed in blood glucose levels. Initial findings appeared to show that the nasal insert formulation was capable of producing similar results to that of a s.c. dose, with an AUC 73.2% compared to s.c., although as the profiles obtained are from one sheep only, such results must be treated with caution.

5.3.2.2 Four way crossover

Blood glucose data was determined as a percentage reduction in the baseline value, and the AUC was determined as the area under the curve compared to the baseline (Table 5.2). Plasma insulin pharmacokinetic parameters (C_{max} and T_{max}) were determined and AUC values were calculated using the trapezoidal rule (Table 5.3).

Table 5.2 Blood glucose data in sheep following nasal administration of 110iu of insulin, compared to 11iu administered subcutaneously.

Dosage Form	Maximum decrease from baseline (%)	T_{max} (hours)	AAC* (%.h)	F_{rel}
s.c.	54.4	1.5	196.0 ±25	100%
Lyophilised insert	11.6	4	59.4 ±50	3.04%
Nasal powder	7.0	5	41.2 ±49	2.11%
Nasal spray	12.0	1.5	63.6 ±46	3.25%

*Area above the curve

Table 5.3 Plasma insulin data in sheep following nasal administration of 110iu insulin, compared to 11iu administered subcutaneously.

Dosage Form	C_{max} (μiu/ml)	T_{max} (minutes)	AUC (μiu.h/ml)	F_{rel}
s.c.	390.0	20	489.0 ±169	100%
Lyophilised Insert	33.3	30	16.9 ±16	0.35%
Nasal Powder	40.1	30	15.6 ±8.8	0.32%
Nasal Spray	83.7	10	37.8 ±20	0.77%

Blood glucose profiles for the four formulations in eight sheep are shown in Figure 5.6, and plasma insulin levels in Figure 5.7. Figure 5.8 shows the plasma insulin profiles of the three nasal formulations alone, to allow a clearer comparison of the profiles.

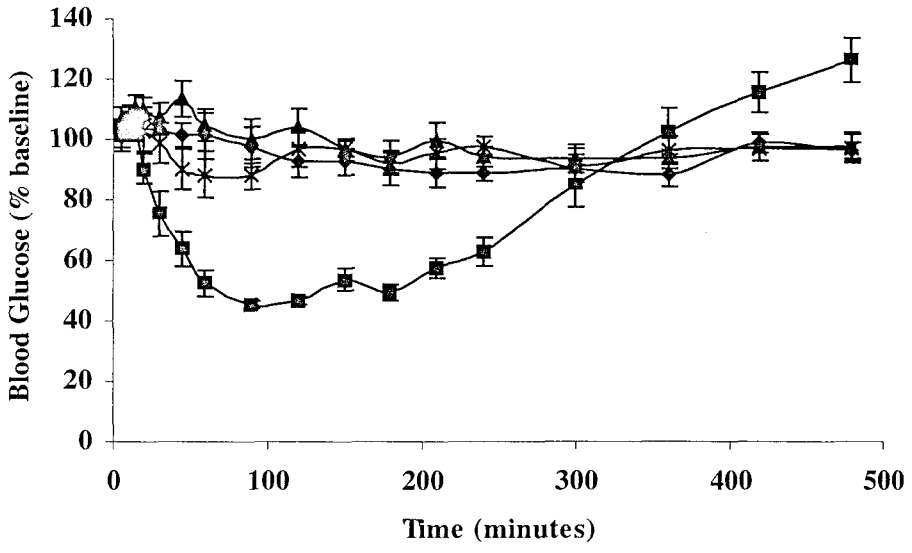


Figure 5.6 **Blood glucose concentration time profiles (expressed as a percentage of the baseline value) of different formulations following intranasal (110iu) and s.c. (11iu) administration of insulin in sheep (n=8).**

(■) s.c. (◆) nasal insert (x) nasal spray (▲) nasal powder.

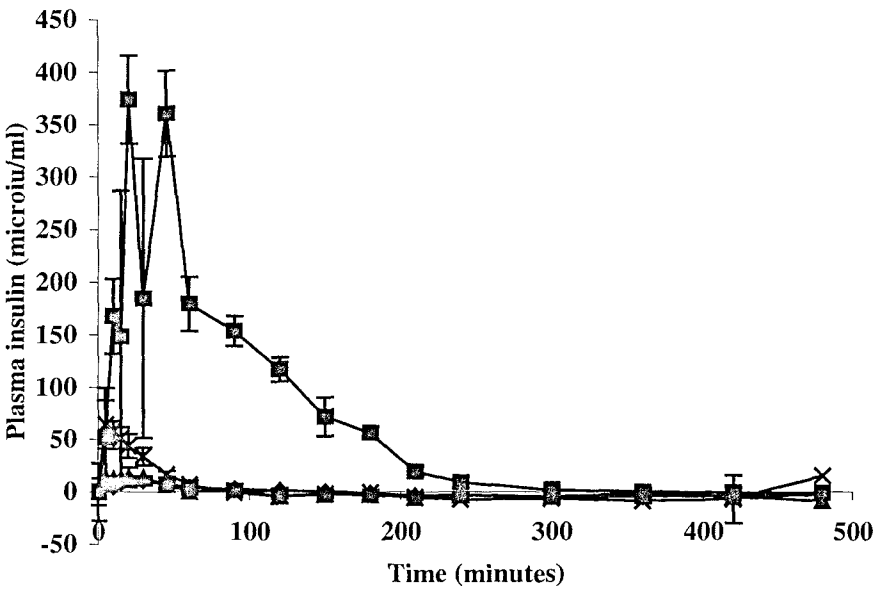


Figure 5.7 **Plasma insulin concentration time profiles (expressed as the increase from the baseline value) of different formulations following intranasal (110iu) and s.c. (11iu) administration of insulin in sheep (n=8).**

(■) s.c. (◆) nasal insert (X) nasal spray (▲) nasal powder.

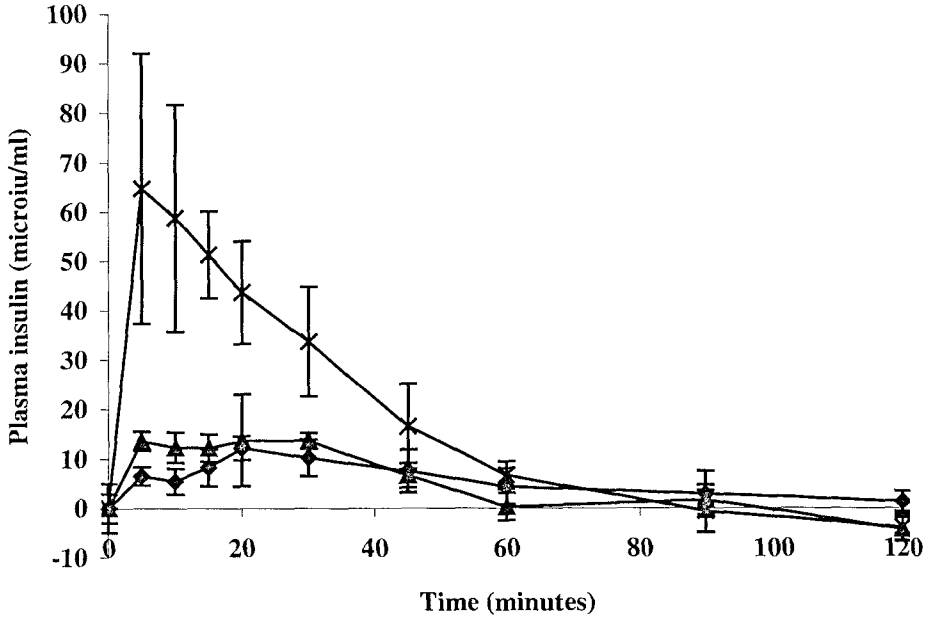


Figure 5.8 **Plasma insulin concentration time profiles expressed as the increase from the baseline value (displayed to 120 minutes for clarity) of different formulations following intranasal (110iu) administration of insulin in sheep (n=8).**

(◆) Nasal insert (x) nasal spray (▲) nasal powder.

The s.c. dose resulted in increased levels of plasma insulin, and a corresponding decrease in blood glucose levels. Modest increases in plasma insulin levels were observed following administration of the nasal doses, with the C_{max} value decreasing in the order spray > powder > insert. However the maximum decrease in baseline plasma glucose levels for the nasal formulations (observed pharmacological effect) decreases in the order spray>insert>powder. The AUC values calculated show a similar pattern. As with data from the NHT study, blood glucose and plasma insulin levels showed high variation, reflected in the standard deviations calculated in Tables 5.2 and 5.3, making statistical analysis difficult.

Nasal formulation plasma insulin AUC's, calculated as a percentage of the s.c. value, were 0.35, 0.77, and 0.32 for the insert, spray and powder respectively, while

as a percentage of blood s.c. glucose AAC's were 3.04, 3.25 and 2.11 respectively. The reason for the lack of correlation of this data is unclear, however when these values are expressed as a ratio of the percentage insulin:percentage glucose, the values obtained are 8.77, 4.25 and 6.6 for the insert, spray and powder, showing that the nasal insert appears to have a greater relative effect on lowering of blood glucose per unit of insulin absorbed than the other nasal formulations, which may be of more physiological relevance, as despite the higher nasal spray insulin levels, the clinical outcome of lowering blood glucose is the ultimate aim of insulin administration. This finding may be explained if a portion of the insulin absorbed from the nasal spray had been in an inactive form, therefore having no effect on blood glucose levels, while the insulin absorbed from the nasal insert was absorbed entirely as a biologically active form. However, when considering and comparing these results, it must be remembered that the nasal doses were ten times that of the s.c. dose.

A possible reason for the results of the pilot study not being reproduced in the four way crossover may have been a change in the method of formulating the inserts. For the pilot study the insulin/HPMC gel was freeze dried at approximately pH 3 (0.01M HCl), whereas in the crossover study the pH of the gel was increased with the addition of NaOH to pH 7. It was therefore thought possible that as the behaviour of insulin is sensitive to pH conditions, this change may have affected the aggregation state of the insulin in the formulation, with resultant decrease in bioavailability. Research has demonstrated that pH can significantly affect nasal absorption of insulin solution in dogs (Hirai et al., 1978), where it was found that a maximum hypoglycaemic effect was demonstrated at pH 3.1, whereas neutral solutions required the addition of absorption enhancers (surfactants) to achieve satisfactory blood glucose levels.

5.3.2.3 Effect of formulation variables

Blood glucose and plasma insulin profiles comparing the nasal formulation variables studied are shown in Figures 5.9-5.11, and a summary of pharmacokinetic parameters is displayed in Tables 5.4 and 5.5. The results are compared with the nasal spray from section 5.3.2.2 as a conventional solution dose, and the 2% K4MP nasal insert which was formulated at pH 7. In general, all nasal formulations had reached a plateau at 300 minutes after dosing.

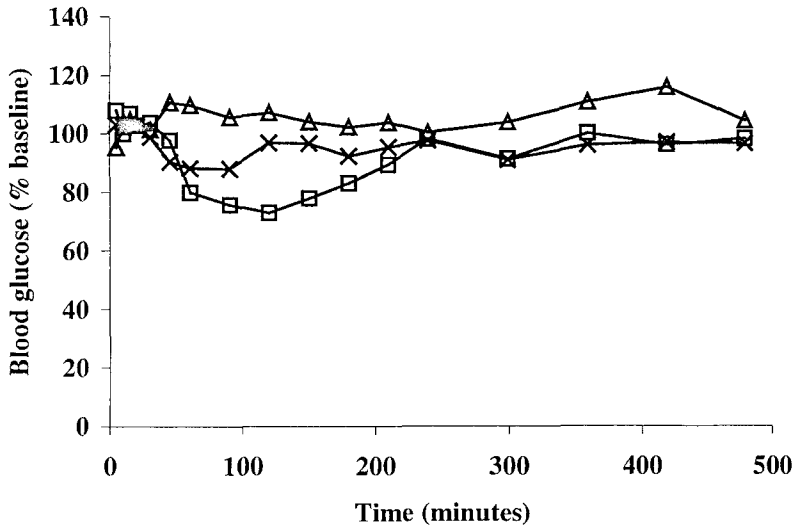
Figure 5.9 shows that the placebo nasal insert had no effect on either blood glucose or plasma insulin, which is reflected in the respective AAC and AUC values of 5.47 %.h and 3.76 μiu.h/ml. The reconstituted nasal insert which was administered as a s.c. dose produced an increase in plasma insulin which lasted for 90 minutes, and gave a corresponding decrease in blood glucose levels between 45 and 240 minutes, demonstrating that the despite the low bioavailability from the original nasal insert formulation, the insulin present had retained a level of activity. The AUC values for the reconstituted nasal insert are much lower than for the conventional s.c. dose (AUC values are 77.8%.h and 43.2μiu.h/mL for blood glucose and plasma insulin respectively, compared with 195%.h and 489μiu.h/mL for the original s.c. dose), an indication that there was however some loss of activity, or perhaps breakdown of insulin. This may have occurred as a result of the freeze-drying process, or possibly even during reconstitution.

It is also interesting to note from the plasma profiles in Figure 5.9 that despite a low level of plasma insulin in comparison with the nasal spray formulation, the effect on blood glucose is much more pronounced, suggesting that the insulin which remains present has retained a higher level of activity than that contained within the spray dose.

Figure 5.10 shows that preparing the 2% K4MP nasal insert at pH 3 had very little effect on blood glucose and plasma insulin levels. Blood glucose was not effectively decreased from baseline values, and the plasma insulin profiles were very similar for both formulations, suggesting that the pH of preparation of the formulation was not a factor in the low bioavailability of the dose.

As can be seen from Figure 5.11, preparing the pH 7 nasal insert formulation with 1% instead of 2% K4MP did not improve nasal absorption, despite the greatly

increased level of overall insulin release which was demonstrated in *in-vitro* studies (Chapter 3). AUC values for blood glucose and plasma insulin were 5.88 and 6.36 respectively for the 1% K4MP formulation, and 59.4 and 16.9 for the 2% K4MP insert, demonstrating that the amount of insulin available for absorption did not appear to be a factor in the low bioavailability of the nasal insert formulation. An explanation for the relative decrease in the AUC's for the 1% K4MP formulation may be that the lower concentration of HPMC in the freeze dried formulation had less of a dehydrating effect on the nasal mucosa, causing less opening of tight cell junctions. This suggests that in this case the osmotic effect of these formulations may play an important role in the absorption enhancing effect, as suggested by Edman et al., 1992.



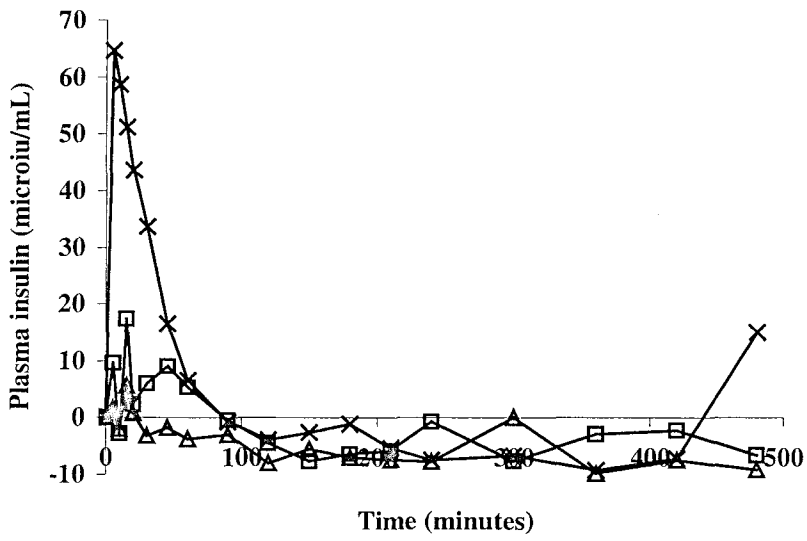
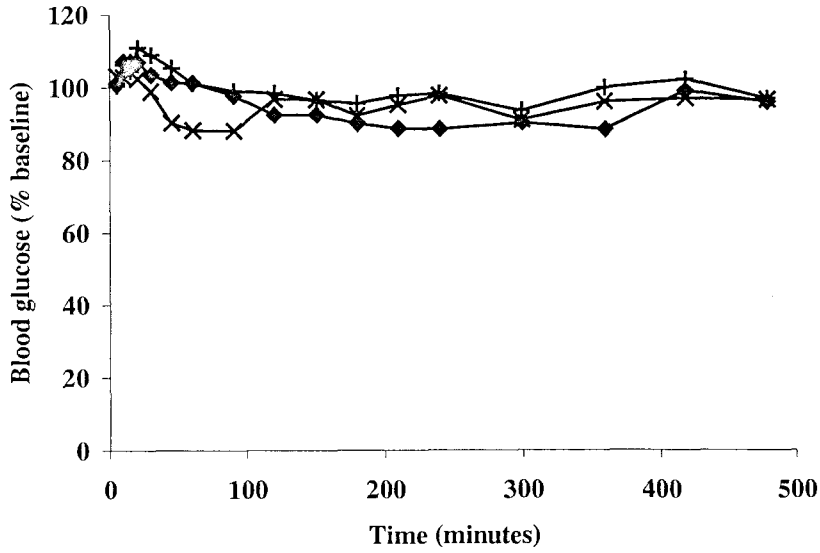


Figure 5.9 **Blood glucose concentration time profiles (expressed as a percentage of the baseline value) and plasma insulin time profiles (expressed as the increase from the baseline value) following intranasal (110iu) and s.c. (reconstituted nasal insert) administration of insulin in sheep (n=8 for nasal spray, n=4 for other formulations).**

(X) Nasal spray (□) s.c. (reconstituted nasal insert) (△) 2% K4MP nasal placebo.



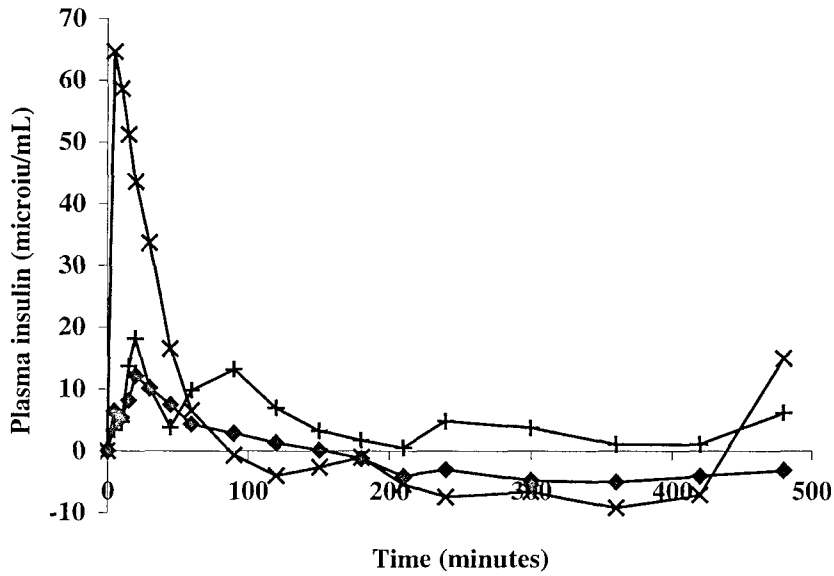


Figure 5.10 **Blood glucose concentration time profiles (expressed as a percentage of the baseline value) and plasma insulin time profiles (expressed as the increase from the baseline value) following intranasal (110iu) administration of insulin in sheep (n=8).**

(x) Nasal spray (◆) 2% K4MP nasal insert pH 7 (+) 2% K4MP nasal insert pH 3.

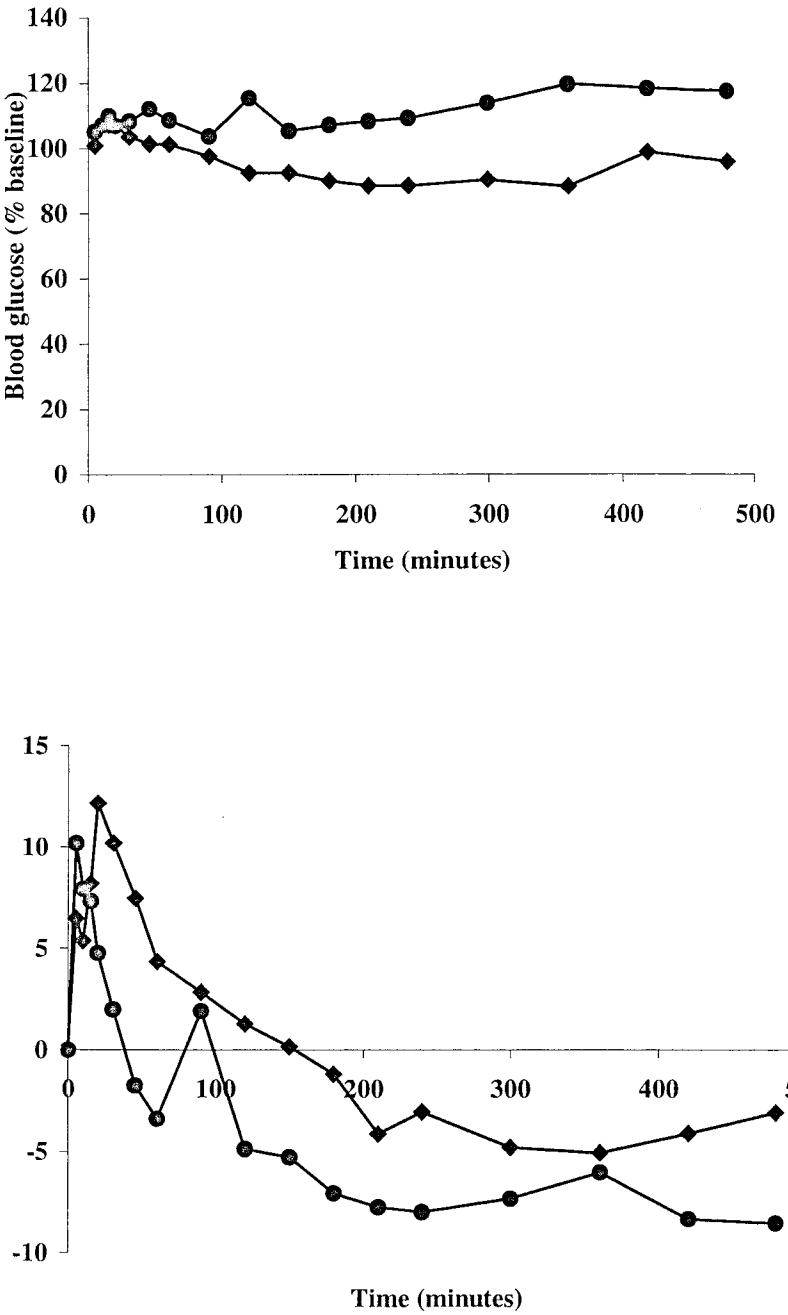


Figure 5.11 **Blood glucose concentration time profiles (expressed as a percentage of the baseline value) and plasma insulin time profiles (expressed as the increase from the baseline value) following intranasal (110iu) administration of insulin in sheep (n=8).**

(◆) 2% K4MP nasal insert pH 7 (●) 1% K4MP nasal insert pH 7.

Table 5.4 Blood glucose data in sheep following nasal administration of 110iu of insulin, compared to 11iu administered subcutaneously.

Dosage Form	Maximum decrease from baseline (%)	T_{max} (hours)	AAC[b] (%.h)	F_{rel}
Placebo	4.68	0.083	5.47 ±50	0.280%
Nasal spray[a]	12.0	1.5	63.6 ±46	3.25%
Reconstituted insert (s.c.)	27.2	2	77.8 ±53	3.98%
2% K4MP nasal insert, pH7[a]	11.6	4	59.4 ±50	3.04%
2% K4MP nasal insert pH3	6.51	5	32.8 ±29	1.68%
1% K4MP nasal insert pH 7	-3.60	1.5	5.88 ±9.5	0.301%

[a]Data from section 5.3.2.2
[b]Area above the curve

Table 5.5 Plasma insulin data in sheep following nasal administration of 110iu insulin, compared to 11iu administered subcutaneously.

Dosage Form	C_{max} (μiu/ml)	T_{max} (minutes)	AUC (μiu.h/ml)	F_{rel}
Placebo	5.65	15	3.76 ±5.7	0.077%
Nasal spray[a]	83.7	10	37.8 ±20	0.765%
Reconstituted insert (s.c.)	17.4	15	43.2 ±43	0.881%
2% K4MP nasal insert, pH7[a]	33.3	30	16.9 ±16	0.346%
2% K4MP nasal insert pH3	18.1	20	52.9 ±68	1.08%
1% K4MP nasal insert pH 7	10.2	5	6.36 ±8.7	0.130%

[a]Data from section 5.3.2.2

T_{max} values tended to be slightly delayed for the nasal insert formulations, with the plasma insulin T_{max} of 5 minutes for the 1% K4MP formulation being earlier than those of 20 and 30 minutes for the pH 3 and pH 7 2% K4MP inserts, as might be expected from a faster releasing, less bioadhesive dose. Some evidence of a 'double peak' effect similar to that described in Chapter 4 for NHT can be observed from the plasma insulin profiles for the 1% K4MP and 2% K4MP pH 3 nasal insert formulations (Figures 5.10 and 5.11), showing that this behaviour is not limited to small molecular weight compounds such as NHT. As in section 5.3.2.2, it must be remembered that ultimately it is the physiologically relevant response of decrease in blood glucose which can be considered the most important outcome following administration of insulin.

The discrepancy between the apparent low AUC on visual examination of the plasma insulin profiles displayed, and higher values displayed in the data tables for the reconstituted nasal insert administered subcutaneously and the pH 3 nasal insert,

is explained in each case by the fact that one sheep exhibited higher plasma insulin levels, which increased the mean value. If the AUC of the mean curve is instead calculated, the values for plasma insulin AUC are 7.29 μiu.h/ml and 36.2 μiu.h/ml respectively. The same is true of the plasma glucose AAC values, which are 71.7 %.h and 12.0 %.h respectively for the mean curve. In the case of the reconstituted insert administered subcutaneously, it is the same sheep which displays uncharacteristically high values, but not for the pH 3 formulation.

Overall, it seems that while the nasal insert formulation produced increased and prolonged nasal absorption of NHT in sheep as demonstrated in Chapter 4, the absorption enhancing effect was not observed with insulin, although evidence of prolonged nasal residence and absorption is observed in the plasma insulin profiles. In addition to those listed above, possible other factors in low absorption include that the molecular weight of insulin is too high for the absorption enhancing effects of this formulation to be applicable, and that the insulin in the formulation may be broken down by enzymes in the sheep nasal cavity (Sarkar, 1992) due to prolonged contact with the nasal mucosa, with the possibility that these enzymes may even diffuse into the hydrating polymer matrix, degrading insulin before it is released.

Interaction of insulin with the formulation may also account for the behaviour of the inserts, despite a report which found that a concentration of 1% mannitol freeze dried with the protein, atrial natriuretic peptide, produced the lowest quantity of multimers (Wu et al., 2000), and that mannitol protected recombinant human growth hormone from aggregation during lyophilisation (Costantino et al., 1998a). A pH dependant interaction of insulin with chitosan nanoparticles (higher at pH 6.1 than pH 5.3) was found to reduce insulin release from the formulation at pH 6.1, but increase enzymatic degradation at pH 5.3 due to 'unfavourable conformational changes' (Ma et al., 2002). A similar possibility of interaction with the polymer of the formulation was proposed by Pereswetoff-Morath and Edman, 1995, who suggested that the hydroxyl groups of the hydrophobic cellulose ether, ethyl (hydroxyethyl) cellulose, may facilitate formation of hydrogen bonds and hydrophobic interaction with the polymer network. This effect may potentially occur with hydroxyl groups in the HPMC polymer.

Further factors which may have influenced results include the proposal that a combination of high insulin concentration at the mucosal surface and its slightly acidic pH may induce aggregation of insulin (Lee et al., 1991), potential effects of the freeze drying process on insulin activity, and that increasing the residual water content of lyophilised insulin may allow plasticisation, increasing the molecular mobility of insulin in the formulation, allowing higher probability of aggregation (Strickley and Anderson, 1997).

5.4 Conclusion

Despite promising findings from the pilot study, results from the larger study suggest that the lyophilised formulation did not result in increased nasal absorption of insulin in sheep in comparison with conventional nasal formulations. However, the ratio of the AUC's of glucose:insulin values relative to s.c. give the impression that the nasal insert has a greater overall effect on the clinical outcome of reduction of blood glucose compared to the nasal powder and spray doses.

Plasma profiles have shown considerable inter- and intra-animal variation, and as a result the mean profiles may be slightly misleading. It is clear however, that previous demonstration of prolonged nasal absorption of NHT from the insert formulation with a corresponding increase in the AUC was not achieved with the administration of insulin.

The blood glucose levels and plasma insulin profiles offered some conflicting information on the effect of the formulation variables. From the results obtained it seems that neither the pH of the formulation, nor the rate of insulin release was responsible for lack of bioavailability of the nasal inserts, although dehydration effects amongst others may be important.

Chapter 6 Suggested Future Work and General Conclusions

6.1 Suggested future work

The following suggestions are made for possible areas of future investigation of the work reported here:

- Section 6.2 outlines preparations which have been made for the investigation of formulation variables on nasal absorption of insulin in human volunteers, and the assessment of nasal residence using scintigraphic imaging. This should provide information on the effectiveness of the *in-vitro* tests and the sheep model in predicting behaviour of the lyophilised nasal insert in man.

- Further examination of the effects of formulation, pH and the freeze drying process on the aggregation of insulin, and its subsequent biological efficacy.

- Investigation of the effects of chemical characteristics and molecular weight of drug on their release profiles from the lyophilisates and *in-vivo* nasal absorption.

- The use of thermal analysis techniques to investigate the amorphous regions detected in the nasal inserts.

6.2 Proposed study in human volunteers

6.2.1 Introduction

Investigation of nasal absorption of insulin in humans has steadily continued over many years, with mixed success. Initial investigations were carried out using commercially available insulin preparations intended for s.c. administration, given intranasally to healthy and diabetic subjects (Pontiroli et al., 1982). Regular (Actrapid) insulin and crystalline insulin (20-60 iu per subject) were mixed with 1% w/v sodium glycocholate as a surfactant, and blood glucose and serum insulin levels determined following nasal administration were compared with an IV dose (0.1iu/Kg) in normal patients, and s.c. dosing in diabetic subjects. It was found that intranasal administration of insulin produced a fall in blood glucose levels, which continued for 52 minutes, and an increase in serum insulin levels.

The relative potency of IN:IV insulin in terms of blood glucose levels was found to be 1:8, although variation in the data was high. It was also found that increasing the concentration of intranasal insulin resulted in higher serum insulin levels, although no direct correlation could be established. A similar study found that intranasal insulin administration resulted in a low bioavailability, and that no statistically significant difference in bioavailability was detected between 100iu/ml or 500iu/mL given as a nasal spray (Valensi et al., 1996).

Other studies describe similarly variable results, using absorption enhancers such as sodium glycocholate (Frauman et al., 1987), di-decanoyl-α-phosphatidylcholine (DDPC) (Jacobs et al., 1993, Drejer et al., 1992, Merkus et al., 1996), Laureth 9 (Valensi et al., 1996, Merkus et al., 1996), sodium taurodihydrofusidate (STDHF) (Merkus et al., 1996), and dimethylated β-cyclodextrin (DMβCD) (Merkus et al., 1996). A summary of results published by some of these researchers is shown in Table 6.1.

Table 6.1 Summary of data from insulin nasal absorption studies in humans.

Absorption enhancer	Insulin dose	Blood glucose (mmol/l)	Insulin C_{max} (miu/l)	Bioavailability (relative to sc)	Reference
DPPC	0.28 iu/Kg	5.0 ±0.3	5.5 ±2.2	16.9/23.9	(Drejer et al., 1992)
	0.59 iu/Kg	4.9 +0.3	4.8 ±1.7		
	0.77 iu/Kg	5.3 ±0.3	4.4 ±2.4		
	50iu	3.6 ±0.7	84.0 ±53	9.9	(Jacobs et al., 1993)
	100iu		190 ±119	11.3	
	150iu		286 ±108	12.3	
9 lauryl ether	135-315iu	1.97-2.33[a]	0.79-1.39	5.14 ±0.4	(Valensi et al., 1996)
Sodium glychocholate	20-60iu	1.9 ±0.3[a]	67.1 ±17	NS	(Pontiroli et al., 1982)

[a]expressed as the maximum decrease in blood glucose
NS Not stated

In general, intranasal doses tended to be about 9 times that which was given subcutaneously.

Arguably one of the most important outcomes from one of these studies, was that six out of the nine diabetic patients taking part in the study expressed a preference for intranasal insulin (Frauman et al., 1987), demonstrating the patient acceptability of this route.

The use of absorption enhancers has been associated with nasal irritation (Merkus et al., 1996), a problem further compounded by the requirement for long-term administration in diabetes, and to overcome these problems bioadhesives have been

investigated to enhance nasal absorption. A basic test frequently used to determine nasal transit is the saccharin test (Guida et al., 2000, Jones, 2001), in which a saccharin tablet is administered nasally to the subject, and the time until a sweet taste is detected is taken as the mucosal transit time. This test can be subjective, as taste perception and sensitivity can vary between individuals. In the assessment of bioadhesive properties *in-vivo*, imaging techniques are generally employed to evaluate the nasal residence of a formulation, and can provide a more accurate measure of mucociliary transport rates.

Gamma scintigraphy is a non-invasive imaging technique which can be used to study mucociliary transit, and to correlate this with pharmacokinetic profiles of the drug. The method involves labelling the dosage form to be studied with a gamma-emitting radioisotope, such as technetium (^{99m}Tc) or indium (^{111}In). Following administration, photons emitted by the radioisotope pass through a collimator, striking a sodium-iodide crystal within the camera (Figure 6.1). The flash of light produced is detected by photomultiplier tubes, with the digital signal produced allowing quantitative analysis of the image produced (Digenis et al., 1998).

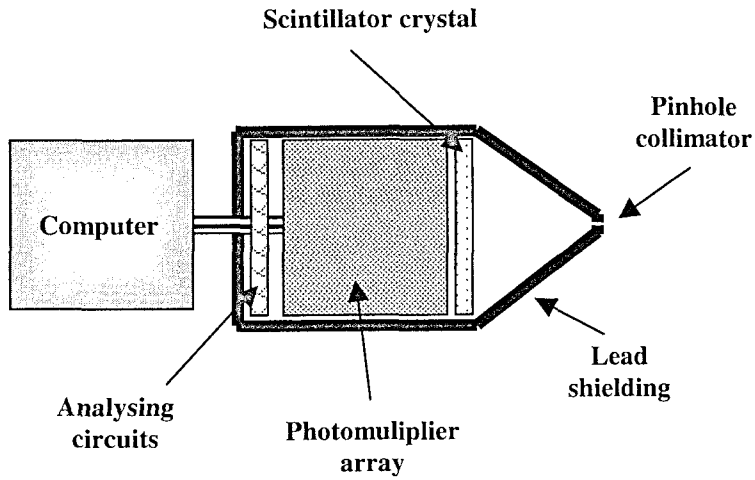


Figure 6.1 Schematic representation of gamma camera.
Redrawn from Meseguer et al., 1994.

Often it is possible to process the images of two different isotopes simultaneously, as they can be separated on the basis of peak photon emissions. Such a double labelling technique was used to image the mucociliary transit time of drug particles and polyacrylic acid microspheres in human subjects (Vidgren et al., 1991), and a sample of the types of images obtained is shown in Figure 6.2.

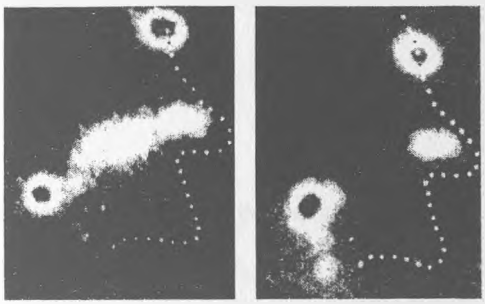


Figure 6.2 Scintigraphic images obtained following nasal administration of desmopressin solution.

(a) nasal spray pump and (b) a rhinyle catheter.

Reproduced from Harris et al., 1988b.

Gamma scintigraphy has been used to elucidate simple transit times of nasal sprays and drops (Bryant et al., 1999, Guida et al., 2000), deposition patterns of nasal sprays (Suman et al., 1999, Eyles et al., 2001, Harris et al., 1988b), effect of posture on deposition (Ridley et al., 1995), administration technique and volume (Newman et al., 1994) and bioadhesive behaviour (Illum et al., 1987, Soane et al., 1999), the technique proving valuable and versatile. For example, the mucociliary transit speed has been calculated as 3.70 ± 0.77 mm/min (Guida et al., 2000), while bioadhesive starch microspheres have been shown to double the nasal transit time from 21 to 41 minutes (Soane et al., 1999).

Gamma scintigraphy has also been used in animal models (Ugwoke et al., 2000a), where it was concluded that the sheep is a suitable model for nasal clearance studies, giving results similar to those in man (Soane et al., 2001), and to assess the relevance of *in-vitro* test in predicting *in-vivo* behaviour (Suman et al., 2000). It was concluded that *in-vitro* spray characteristics tested did not predict deposition patterns *in-vivo*, demonstrating the importance of *in-vivo* imaging techniques.

6.2.2 Materials and Methods

6.2.2.1 Chemicals

For the *in-vitro* release study, HPMC powder (K4MP grade) was obtained as a gift from Dow Chemicals (Michigan, USA) and D (-) mannitol powder (GPR) was purchased from BDH (VRW, Poole, UK). Insulin (bovine) and phosphate buffered saline (PBS) pH 7.4, were purchased from Sigma (Gillingham, UK). Bio-Rad Protein Assay was purchased from Bio-Rad Ltd. (Hemel Hempstead, UK). Decayed radioisotopes Technetium (^{99m}Tc) and Indium (^{111}In) were supplied by Bio-Images, Glasgow, UK.

For the proposed human nasal scintigraphy and absorption study all materials had a Certificate of Analysis stating that they were of BP grade or equivalent or of USP grade in the case of K4MP powder, obtained as a gift from Dow Chemicals (Michigan, USA). BP grade D (-) mannitol powder, insulin powder (human), concentrated HCl, and NaOH pellets were purchased from Sigma (Gillingham, UK). Water for Injection was obtained from Baxter Healthcare (Glasgow, UK).

6.2.2.2 Apparatus

Polythene microcentrifuge tubes (0.2mL) were obtained from Life Sciences, UK. Whatman Filter Paper (Grade 1, 42.5mm diameter) was purchased from BDH (VRW, Poole, UK). Small volume (700μL) far UV quartz spectrophotometer microcells of path length 1cm were purchased from BDH (VRW, Poole, UK). A UV double beam spectrophotometer (Helios Alpha or UV1, Thermospectronic, Rochester, USA.) was used in the analysis of insulin content of samples using the BioRad Protein Assay. A Virtis Advantage Freeze Drier, which carried out a cycle with preset stages, was used to lyophilise HPMC gels (BioPharma Process Systems, UK).

6.2.2.3 Methods

6.2.2.3.1 Preparation of HPMC gels for *in-vitro* insulin release

HPMC gels containing insulin were prepared as described in section 2.2.3.1, with the addition of 20μL of decayed radioisotope added to each 0.26mL of the gel.

6.2.2.3.2 Preparation of lyophilised formulations for *in-vitro* insulin release

HPMC gels were lyophilised as described in section 2.2.3.2.

6.2.2.3.3 *In-vitro* release of insulin from radioisotope containing nasal inserts

The *in-vitro* release of insulin from lyophilised nasal inserts containing ^{99m}Tc or ^{111}In was assessed using the method outlined in section 3.2.3.4.

6.2.2.3.4 Manufacture of insulin containing lyophilised inserts with indium for proposed human nasal absorption investigation

The manufacturing protocol for the 2% K4MP lyophilised nasal inserts by qualified research centre staff is shown in Appendix B. Manufacturing protocols for 1% and 3% K4MP nasal inserts differ only in the weight of HPMC to be used. The manufacturing protocols were devised by the author, who will be involved in an advisory role.

6.2.2.4 Study protocol

The study protocol given ethical approval is shown in Appendix C. The radioisotope used will be ^{111}In, as due to the length of time the formulation must remain in the freeze drier, the short half life of ^{99m}Tc means it would decay too rapidly.

The information leaflet to be provided to patients regarding the study is shown in Appendix D, and the patient consent form is shown in Appendix E.

The author provided the background information for ethical application (Appendix C), was involved in discussions of the overall design of the study, recruitment of potential volunteers, and was responsible for obtaining all equipment

and chemicals other than radioactive isotopes, which are to be provided by Bio-Images (Glasgow, UK). All other aspects of the application for ethical approval and organisation of the study were undertaken by the Bio-Images staff.

6.2.3 Results and Discussion

6.2.3.1 *In-vitro* release of insulin from lyophilised inserts containing ^{99m}Tc

Release profiles from the insulin loaded lyophilised inserts containing ^{99m}Tc are shown in Figure 6.3. As in the results displayed in chapter 3 for insulin release with no isotope present, the rate of insulin release from the lyophilised preparations decreased in the order 0.5% K4MP > 1% K4MP > 2% K4MP > 3% K4MP, with little or no insulin release until 30 minutes.

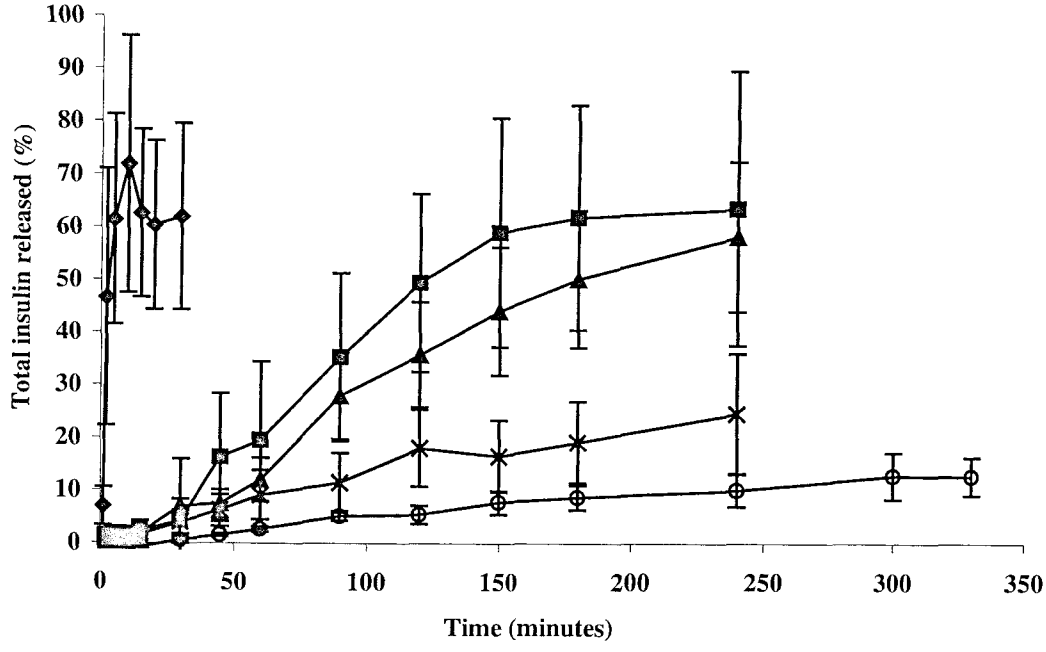


Figure 6.3 **Profiles showing release of insulin from formulations containing 49iu insulin and ^{99m}Tc.**

(◆) insulin solution, (■) 0.5% K4MP lyophilised insert, (▲) 1% K4MP lyophilised insert, (x) 2% K4MP lyophilised insert, (○) 3% K4MP lyophilised insert.

6.2.3.2 *In-vitro* release of insulin from lyophilised inserts containing ^{111}In

Release profiles from the insulin loaded lyophilised inserts containing ^{111}In are shown in Figure 6.4.

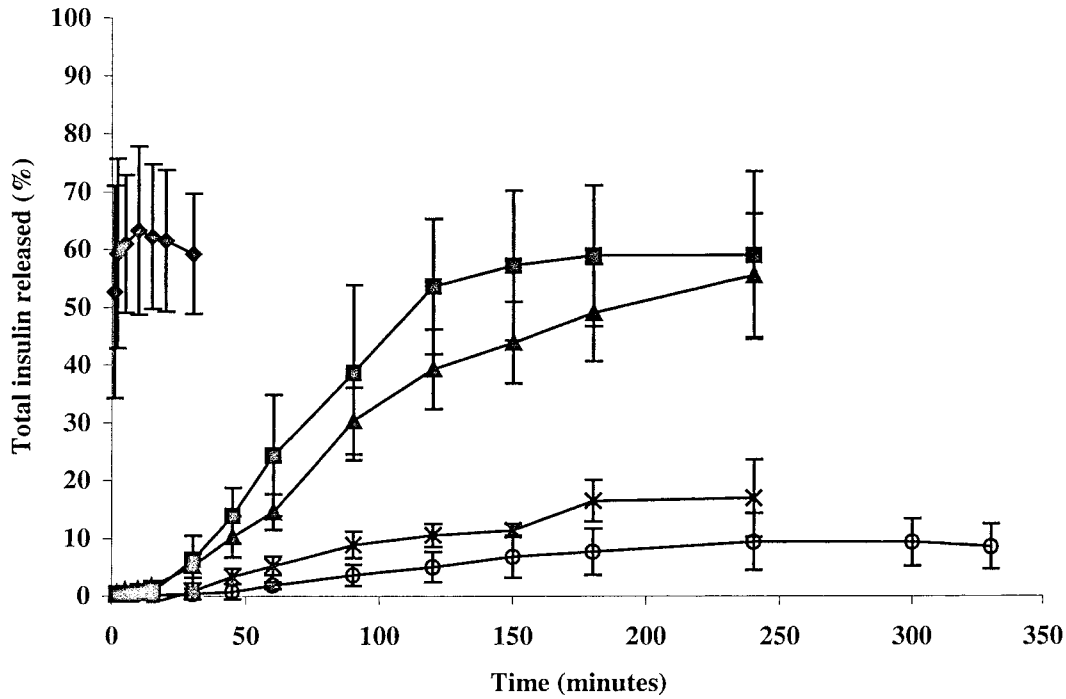


Figure 6.4 **Profiles showing release of insulin from formulations containing 49iu insulin and ^{111}In.**

(◆) insulin solution, (■) 0.5% K4MP lyophilised insert, (▲) 1% K4MP lyophilised insert, (x) 2% K4MP lyophilised insert, (○) 3% K4MP lyophilised insert.

The insulin release profiles obtained for the radioisotope containing inserts were very similar, and were also similar to the release profiles displayed in Chapter 3. As described in Chapter 3, the gradient of the linear phase of the release profiles was used as a means of comparing the release behaviour of the different formulations (Table 6.2).

Table 6.2 Comparison of slope of linear phase of release between lyophilised insert formulations containing K4MP, 49iu insulin and ^{99m}Tc or ^{111}In.

K4MP concentration	Formulation		
	Insulin	Insulin and ^{99m}Tc	Insulin and ^{111}In
0.5%	0.642	0.416	0.474
1%	0.456	0.310	0.339
2%	0.217	0.147	0.107
3%	0.050	0.045	0.042

The results obtained from the insulin release studies show that despite some variation in the data, leading to slight differences between the gradient of the linear phase of release, in general the profiles obtained are very similar, suggesting that the inclusion of either isotope does not significantly alter the release of insulin from the formulation.

6.2.3.3 Proposed human nasal absorption investigation

To date, the proposed study has not been carried out due to a lack of adequate insurance cover.

6.2.4 Conclusion

Pending provision of insurance cover, the clinical study in humans is expected to provide vital information on the behaviour of the lyophilised nasal insert when administered to human subjects. The analysis of scintigraphic data will allow visualisation of the mucosal transit of the rehydrated insert, elucidating the extent of spreading in the nasal cavity and any bioadhesive properties *in-vivo*, and the effect of K4MP concentration on this parameter.

Mucociliary transport rates can be calculated for the different formulations, which may then be compared with the pharmacokinetic data in an attempt to rationalise profiles obtained. Scintigraphic determination of nasal residence of the inserts will also be useful in providing further evidence for a bioadhesive effect producing the prolonged absorptive phase observed with NHT in sheep in Chapter 4. Blood glucose levels and plasma insulin will provide information on the nasal absorption of insulin from the lyophilised inserts in humans, and how any insulin absorption translates into a clinical outcome.

With the study of three different K4MP concentrations, would come the essential opportunity to compare the effect of formulation on *in-vitro* release profiles and *in-vivo* nasal absorption, to determine if the *in-vitro* test can in future be used to predict behaviour *in-vivo*, allowing optimisation of formulation before *in-vivo* tests are carried out.

Clinical data from the proposed study in human volunteers could also be compared with the plasma insulin and blood glucose levels determined in sheep for the lyophilised insert, to elucidate any possible correlation between the behaviour of the insert in the sheep model and man.

6.3 General Discussion

The *in-vitro* and *in-vivo* properties of a lyophilised HPMC formulation were examined to assess its potential as a nasal bioadhesive dosage formulation. Textural analysis was initially used to assess adhesion to a synthetic mucosal surface (agar), but results obtained were variable, and differences between formulations were not easily detected. As a result, two methods were developed to more accurately reflect the motion of a bioadhesive as it is transported across the mucosal surface by the nasal cilia. Dynamic adhesion tests produced a dynamic adhesion value which could be used to compare formulations, and demonstrated that increasing polymer molecular weight and concentration resulted in increased adhesion, while increasing the concentration of the excipient mannitol did not. A TA sliding adhesion test demonstrated that an increased level of hydration resulted in a gradual decrease and plateau in adhesion.

Imaging using the CLSM proved a useful technique to calculate differences in the rates of hydration of lyophilised formulations, demonstrating an initial rapid rate water ingress, followed by a steady state rate of hydration which occurs after a few minutes. Insulin release profiles were determined using a dissolution method designed to mimic the conditions of the nasal cavity, and demonstrated that following an initial linear phase of insulin release, increasing the concentration of polymer resulted in a decrease in the rate and total quantity of insulin released.

DVS studies demonstrated the high water vapour sorption capacity of the lyophilisates, and that the presence of NHT in the formulation significantly increased this effect, although there was no evidence of a linear polymer or mannitol concentration dependant effect. A recrystallisation event detected during DVS studies was assumed to be due to sorption of water vapour lowering the glass transition temperature, and plasticising amorphous mannitol, allowing sufficient molecular mobility for crystallisation to occur. The quantity of amorphous mannitol appeared greatly increased in the presence of NHT, and SEM images appeared to provide both evidence of a change in surface structure of the lyophilisates in the presence of NHT, and of an effect of relative mannitol concentration on the crystal structure formed.

SEM images also showed the highly porous internal structure of the lyophilisates, and allowed determination of a polymer concentration dependant decrease in pore size, which correlated with both the rate of water ingress calculated during CLSM studies, and the initial linear release phase of insulin determined in dissolution studies.

In sheep nasal absorption studies, it was shown that the nasal insert formulation produced increased bioavailability and prolonged plasma NHT levels when compared with conventional nasal powder and spray formulations. Evidence of a "double peak" effect was observed in plasma NHT levels, thought to be due to an initial dehydrating effect of the formulation on the nasal mucosa opening tight cell junctions and allowing NHT released to cross the nasal mucosa, followed by a more prolonged phase of NHT release and absorption as the formulation hydrates and spreads over the nasal mucosa. This theory is compatible with the finding from the CLSM study that the lyophilisates show an initial rapid rate of water ingress, followed by a slower steady rate of hydration.

Nasal absorption of insulin from the lyophilised insert in sheep was not enhanced in comparison with conventional nasal preparations, despite attempts to improve bioavailability by altering the pH of the formulation, or reducing the polymer concentration to a level that demonstrated increased insulin release *in-vitro*. In fact, results demonstrated that the formulation with a higher HPMC concentration gave rise to slightly increased plasma insulin levels, further suggesting that the dehydration effect on the nasal mucosa may play an important role in increasing nasal absorption, as a higher polymer concentration would be expected to produce this effect to a greater extent. During the course of the nasal insulin absorption studies, further evidence of the "double peak" effect on plasma insulin levels was observed.

In conclusion, a combination of adhesion, hydration and physical analysis has provided invaluable *in-vitro* data on the effects of formulation on the properties of the lyophilised nasal inserts, while sheep studies have provided information on the *in-vivo* behaviour of the dosage formulation, and the nasal absorption of two different drugs.

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Appendix A

In-vivo plasma nicotine data in sheep

IV (1mg)

Time	Sheep plasma nicotine (ng/ml)							
(min)	1	2	3	4	5	6	7	8
0	0	0	0	0	0	0	0	0
2	15.29	15.01	9.608	4.017	4.010	15.66	14.50	5.419
5	17.61	14.46	8.410	*2.404*	*1.639*	11.69	9.028	6.312
10	9.785	12.06	7.678	*-0.090*	*0.142*	4.757	4.896	*2.444*
15	10.22	11.16	5.502	*0.507*	*0.010*	5.254	4.603	*2.354*
20	5.348	9.408	6.285	*-0.377*	*1.186*	4.104	4.651	*1.746*
30	13.76	9.325	4.282	*-0.665*	*3.449*	5.097	*3.131*	*0*
45	13.88	4.008	*3.305*	*-1.529*	*0.929*	*0*	*4.369*	*0*
60	5.992	9.220	*2.045*	*-1.280*	*2.012*	*0*	*5.572*	*0*
90	8.742	7.231	*1.890*	*-1.557*	*0.076*	*0*	*5.896*	*0*
120	7.122	8.801	*2.788*	*-2.122*	*-0.810*	*0*	*4.421*	*0*
180	13.56	5.766	*2.977*	*-0.479*	*-2.331*	*0*	*2.253*	*0*
240	23.37	8.518	*0*	*-1.337*	*0.388*	*0*	*2.585*	*0*
360	8.795	7.767	*3.041*	*-1.436*	*0.777*	*0*	*2.821*	*0*
480	6.481	5.974	*3.170*	*0*	*0*	*0*	*3.208*	*0*

(Data in italics denotes values excluded due to falling below the 4ng/mL limit of quantitation)

Nasal insert

Time	Sheep plasma nicotine (ng/ml)							
(min)	1	2	3	4	5	6	7	8
0	0	0	0	0	0	0	0	0
15	14.35	*3.733*	12.10	20.35	14.43	24.76	62.41	27.00
30	13.37	*4.084*	*1.592*	7.883	10.80	4.140	26.280	*3.002*
45	19.32	*3.085*	*9.592*	7.936	5.394	*3.520*	83.55	*1.892*
60	13.67	*3.673*	*-1.961*	9.054	5.268	*2.426*	64.54	*1.007*
90	25.04	*16.85*	*0.262*	6.816	6.814	*2.911*	78.37	*-0.398*
120	12.87	*1.845*	*-0.935*	9.325	4.841	*2.425*	25.64	*0.240*
180	*2.190*	*3.399*	*-2.965*	10.13	*3.116*	*3.115*	17.96	*0.986*
240	*2.053*	*1.199*	*-0.281*	9.962	*3.686*	*1.868*	9.790	*-0.037*
360	*2.922*	*1.776*	*-1.328*	7.125	*1.510*	*3.372*	7.866	*1.232*
480	*2.802*	*0.590*	*-0.617*	8.484	*1.795*	*1.935*	6.450	*0.120*

(Data in italics denotes values excluded due to falling below the 4ng/mL limit of quantitation)

Nasal spray

Time	Sheep plasma nicotine (ng/ml)							
(min)	1	2	3	4	5	6	7	8
0	0	0	0	0	-	-	0	0
15	62.69	146.2*	52.12	420.0*	-	-	40.64	66.13
30	26.01	-	6.126	5.713	-	-	25.84	5.297
45	18.41	7.137	5.243	6.458	-	-	8.416	4.226
60	6.141	13.57	*2.914*	*0*	-	-	*0*	*3.968*
90	5.117	*0.177**	*3.194*	*0*	-	-	*0*	*3.453*
120	9.904	14.89	*3.627*	*3.114*	-	-	*0*	*4.636*
180	5.900	8.685	*4.047*	*0*	-	-	*0*	*4.607*
240	6.711	12.38	*3.867*	*0*	-	-	*0*	*3.025*
360	6.729	11.55	*4.625*	*2.639*	-	-	*0*	*4.223*
480	4.571	*3.623*	*0.935*	*0*	-	-	*0*	*3.977*

'-' denotes missing data.
* abnormal values excluded from analysis.
(Data in italics denotes values excluded due to falling below the 4ng/mL limit of quantitation)

Nasal powder

Time	Sheep plasma nicotine (ng/ml)							
(min)	1	2	3	4	5	6	7	8
0	0	0	0	0	0	-	0	0
15	18.24	30.62	34.39	60.62	4.120	-	53.92	5
30	14.42	39.90	12.93	*3.145*	11.06	-	40.33	*0*
45	5.336	17.38	6.758	5.659	5.225	-	10.64	*0*
60	*2.313*	46.08	5.428	*2.153*	4.164	-	4.490	*0*
90	*3.158*	*-0.545*	*3.143*	*1.447*	*3.277*	-	*2.492*	*0*
120	*3.797*	*-1.351*	*5.492*	*2.101*	*3.637*	-	*-0.664*	*0*
180	*2.221*	*0.003*	*5.589*	*3.310*	*3.095*	-	*-0.788*	*0*
240	*1.887*	*-2.339*	*5.834*	*2.721*	*2.677*	-	*-0.357*	*0*
360	*2.516*	*-1.826*	*5.212*	*1.470*	*2.500*	-	*-0.140*	*0*
480	*0.823*	*-1.497*	-	*1.339*	*2.876*	-	*-0.058*	*0*

'-' denotes missing data.
(Data in italics denotes values excluded due to falling below the 4ng/mL limit of quantitation)

Appendix B

Manufacturing Protocol

MANUFACTURE OF GEL

	Tick or Record Result	Procedure by/Date	Checked by/Date
1. Weigh beaker to contain insulin gel and record weight.			
2. Calculate final weight of beaker + contents (weight from step 1. + 10.140g).			
3. Record weight of weighing boat.			
4. Calculate weight of weighing boat + 66.82mg insulin.			
5. Weigh insulin into weighing boat. Record actual weight of insulin added.			
6. Transfer insulin to beaker. Use three or four drops of 0.05M HCl to wash powder from weighing boat into beaker.			
7. Add 0.1mL drops of 0.05M HCl to the insulin in the beaker using Pipetman pipette, stirring with spatula after each drop, until the solution becomes clear.			
8. Add dropwise amounts of 0.1M NaOH until the pH of the solution is pH7.			
9. Weigh new weighing boat.			
10. Calculate weight of weighing boat + 101.4mg mannitol.			
11. Weigh 101.4mg of mannitol into weighing boat. Record actual amount of mannitol added.			
12. Add the mannitol to the insulin solution, using three or four drops of water to wash the remaining powder into the beaker, and stir until the mannitol is fully dissolved.			
13. Weigh new weighing boat.			
14. Calculate weight of weighing boat + 202.8mg K4MP.			
15. Weigh 202.8mg of K4MP into weighing boat. Record actual amount added.			

MANUFACTURE OF GEL

	Tick or Record Result	Procedure by/Date	Checked by/Date
16. Add the K4MP to the insulin and mannitol solution (do not stir).			
17. Add distilled water to the contents of the beaker until the weight equals the amount recorded in step 2.			
18. Stir the solution using the overhead stirrer for 30 to 40 minutes, occasionally stopping to ensure that there are no lumps of HPMC stuck to the paddle, or the bottom of the beaker. The spatula can be used to loosen any lumps, and also to aid dispersion of small clumps of material. The solution should be uniform throughout.			
19. Refrigerate gel for about six hours, or until all the air bubbles have disappeared.			
20. Remove gel from fridge and allow to stand for 30 minutes to come to room temperature.			
21. Add required amount of Indium (add calculation), and stir gently using spatula, being careful not to reintroduce air bubbles into the solution.			
22. Using Microman, measure gel into 0.2mL Eppendorf tubes.			
23. Freeze dry the gels in the Eppendorfs on recipe Fiona 1.			
Comments:			

Appendix C

Study Protocol

Bio-*i* mages

PROTOCOL version 1.0,

PROTOCOL NUMBER:	BC-028-01(v1)
STUDY TITLE:	Study to compare the nasal residence and absorption of insulin from 3 nasal inserts and a nasal solution in healthy male volunteers
INVESTIGATOR(S):	
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Principal Medical Investigator:	Dr J Petrie
Clinical Research Manager:	Dr B O'Mahony
Clinical Project Manager:	Dr B Lindsay
Nuclear Medicine Technician:	Ms T Jones
Research Student:	Ms F McInnes
CLINICAL CENTRE:	Bio-Imaging Centre, Glasgow Royal Infirmary, Glasgow, G4 0SF
ADVISORY PHYSICIANS:	Dr J Petrie Dr C Perry Glasgow Royal Infirmary

STUDY PERSONNEL

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STUDY SUMMARY BC-028-01(v1)

Protocol Title:	Study to compare the nasal residence and absorption of insulin from 3 nasal inserts and a nasal solution in healthy male volunteers
Objective:	To compare the nasal residence and distribution of 3 novel nasal insulin inserts with a nasal insulin solution To compare the bioavailability of insulin delivered from 3 novel nasal insulin inserts with a nasal insulin solution
Study Design:	Single centre, open-label, non-randomised cross-over study
Active Ingredient:	Human insulin
Treatment:	Treatments will be administered in the following sequence: Nasal solution containing 49IU human insulin 1% Lyophilised HPMC nasal insert with 49IU human insulin 2% Lyophilised HPMC nasal insert with 49IU human insulin 3% Lyophilised HPMC nasal insert with 49IU human insulin
Assignment:	Non randomised
Population:	Healthy male volunteers aged between 18-60 years inclusive
Sample size:	8 subjects
Adverse Events:	Volunteered and elicited
Data Analysis:	Scintigraphic analysis of nasal residence Blood glucose Plasma insulin concentration
Proposed Start Date:	January 2002

CONTENTS

1 INTRODUCTION AND RATIONALE

The nasal route of administration has been receiving increasing attention as an alternative route of delivery, particularly for drugs which are currently administered by often painful injections. The nasal mucosa has a rich vasculature allowing the opportunity for improved absorption of compounds, and avoidance of the hepatic first pass clearance effect, as drugs are absorbed directly into the bloodstream. This provides an obvious potential alternative route of administration for drugs which display poor oral absorption due to problems such as low bioavailability, high hepatic first pass clearance and metabolism in the GI tract. The large number of nasal preparations currently available on the market demonstrates patient acceptability of this route.

Proteins and peptides are of particular interest as they are generally currently administered via injection, and present a challenge for nasal delivery, in terms of factors such as the size of the molecule and low absorption due to the rapid mucocilliary clearance rate of the nasal cavity ($t_{1/2}$ approximately 12-15 minutes (*Martin et al*)). In an attempt to overcome these difficulties many researchers use absorption enhancers, with the aim of improving the nasal absorption of the peptide in the brief period that the formulation will be in contact with the nasal cavity. Bile salts (*Natsume et al*), cyclodextrins (*Schipper et al*), fusidate derivatives (*Longenecker et al*), phosphatidylcholines (*Illum et al 1990*) and chitosan salts (*Illum et al 1994*) are among the excipients reported as being used. However the use of these absorption promoters has often been found to result in some damage to the nasal mucosa or the function of the cilia (*Gizurarason et al*), and may not be considered suitable for long term use.

A second method commonly used, either alone or in combination with absorption enhancers, is bioadhesion. Bioadhesive substances essentially "stick" to the nasal mucosa, slowing down the rate of mucocilliary clearance and allowing an extended period of contact with the nasal mucosa for the formulation. In this way it is hoped that increased absorption will be achieved, particularly for large compounds such as peptides which may take longer to permeate the mucosa. It is speculated that one of the mechanisms of action of bioadhesive formulations is a dehydrating effect on the cells of the nasal mucosa, resulting in a transient opening of the junctions between the cells. This may aid absorption of larger molecules across the nasal mucosa.

HPMC gels are bioadhesive compounds which are commonly used as thickening agents and as tablet excipients. HPMC is classified as GRAS (generally recognised as safe by the FDA). Nasal administration of HPMC as a powder or gel to increase nasal residence time has been reported previously. However gels can be difficult to administer, and there will be a limit to the concentration of gel that can be formulated. The formulation of a freeze dried nasal insert offers a dosage form which may be easier to administer due to its ability to be handled. On contact with the nasal mucosa the lyophilate should rehydrate, forming a gel of a higher concentration of HPMC than could be achieved or administered normally. It is hoped that this

rehydrated, concentrated HPMC gel would therefore offer increased bioadhesion and therefore residence time in the nasal cavity.

Theoretically, the combination of the properties of extended nasal residence, and potential enhancement of absorption of large molecules should be of benefit in administration and promotion of absorption of drugs such as peptides.

A previous *in vivo* study in sheep showed promising results with nasal absorption of nicotine from a lyophilised HPMC gel insert. Administration of nicotine as a nasal powder and a nasal solution resulted in similar absorption profiles to that of an IV dose of nicotine. The absorption of nicotine was rapid, with a T_{max} of approximately 15 minutes (solution and powder), and very little plasma nicotine was shown after one hour. A completely different plasma profile was obtained from the lyophilised insert. The T_{max} for the formulation was approximately 42 minutes, and an extended absorption profile was observed, with steady plasma nicotine levels over a period of approximately three hours. The AUC value obtained for the nasal insert was approximately double that of both the powder and the solution. The results from this study would appear to suggest that the lyophilised formulation acts to promote absorption of nicotine without the need for any other form of absorption enhancer.

In the current study it is proposed to investigate the nasal distribution and residence of the insert formulation using scintigraphy. Combining scintigraphy with pharmacokinetic analysis of a model drug will help elucidate the mechanism of action of the formulation. Insulin is an ideal model compound to use in further studies of these inserts as it is a drug which is currently administered to patients via subcutaneous (s.c.) injection, and therefore could potentially have a clinically relevant application of benefit to many patients. Nasal administration may offer a patient acceptable alternative to frequent self-injection. Attempts to deliver insulin nasally with the use of absorption enhancers have been reported more frequently in the literature that with other peptides, therefore offering a comparison for any results obtained.

2 OBJECTIVES

To compare the nasal residence and distribution of 3 novel nasal insulin inserts with a nasal insulin solution
To compare the bioavailability of insulin delivered from 3 novel nasal insulin inserts with a nasal insulin solution

3 STUDY DESIGN

This is a single centre, open label, non-randomised crossover trial in 8 healthy male volunteers aged between 18 and 60 years inclusive. Dropouts will not be replaced

4 ETHICAL CONSIDERATIONS

Local Ethics Committee approval will be sought and obtained prior to the initiation of the trial. Informed consent will be obtained from each patient following written and verbal explanation of the study prior to entry into the trial.

5 STUDY POPULATION

8 healthy male subjects aged between 18 and 60 years inclusive who satisfy the following selection criteria will be entered into the study. Prior to screening written informed consent from each subject will be obtained.

Inclusion Criteria

- Male
- Aged between 18-60 years inclusive
- Prepared and able to give written informed consent
- Normal medical history particularly with reference to nasal cavity.
- Satisfactory mucociliary clearance determined by saccharin test
- Satisfactory medical examination particularly with reference to the nasal cavity
- Body mass index within the range 19 to 28 inclusive

Exclusion Criteria

- Participation in any clinical study/trial during the three calendar months prior to the study or four studies within the previous 12 months
- Subject whose participation in this study results in a total blood donation of greater than 1500ml within the previous 12 months
- Subject who will exceed the limits of total radiation exposure (5mSv) allowed in any 12 month period.
- Subject is diabetic
- Subject with recent (14 days) upper or lower respiratory tract infection
- Subject who suffers from nasal allergies e.g. allergic rhinitis
- Subjects with significant concomitant medication
- Subject has an alcohol intake of greater than 30 units per week
- Subject is a smoker
- Subject with known sensitisation to any components of the dosage form

6 TREATMENTS

Treatments will be administered in the following sequence:

Nasal solution containing 49IU human insulin
1% Lyophilised HPMC nasal insert with 49IU human insulin
2% Lyophilised HPMC nasal insert with 49IU human insulin
3% Lyophilised HPMC nasal insert with 49IU human insulin

Each formulation will contain 4MBq technetium 99m DTPA

7 STUDY PROCEDURES

7.1 PRESTUDY MEDICAL SCREENING

All volunteers will be given a medical examination at the BioImaging Centre during the three weeks before dosing to establish their fitness to participate in the study. This will include a medical history, physical examination, and the assessment of blood pressure and pulse. Blood samples will be taken for laboratory safety assessments, which include serum biochemistry, haematology and hepatitis B and C screens. Additionally, a urine sample will be taken for urinalysis. The results of all screening procedures will be documented in the Case Report Form (CRF). If, in the opinion of the physician, any volunteer has any clinically significant abnormalities in any of the assessments, including the laboratory safety tests, they will be excluded from the study.

7.2 STUDY DAY RESTRICTIONS

Subjects will be asked to comply with the following restrictions before each study day:

- Significant concomitant medication within 14 days prior to study day
- Fasting from 22.00hrs the previous day

Subjects will be questioned on compliance with restrictions prior to commencement of each day. The Investigator will determine if a deviation from the restrictions warrants that subjects withdrawal from the study. Any deviations will be documented in the CRF.

7.3 PRESTUDY PROCEDURES

Subjects who meet the study criteria after the initial screening will attend the study centre 30 minutes prior to dosing in order to acclimatise. All subjects will remain in the department during each study period.

Intravenous access will be established by inserting a cannula into a suitable vein in one of the subjects arms.

A radioactive marker will be attached to the subjects temple

7.4 DOSING

At time zero, the formulation will be inserted into one nostril only. The nostril to be dosed will be randomised and the same nostril will be used on each subsequent occasion. The subject will be seated at the camera during dosing with the dosed nostril closest to the camera. The nasal solution will be administered using a disposable nasal dropper. The nasal insert will be inserted using a specialised device.

7.5 SCINTIGRAPHIC IMAGING

Subjects will be seated laterally in front of a low energy high resolution collimator.

Imaging will begin at time of dosing. A dynamic scan will record 5 second frames for a 3 minute period then 15 second frames up to10 minutes. Subsequent 60 second static views will be collected every 5 minutes for a period of 30 minutes then every 15 minutes until activity has cleared from the nasal cavity.

An outline of the head will be traced on one image using a radioactive pen

7.6 BLOOD GLUCOSE MONITORING

The volume of blood removed for glucose monitoring will be 1ml per sample including dead volume. Blood glucose will be analysed in whole blood on site using a Yellow Springs automated glucose analyser.

Two consecutive samples will be taken within 10 minutes prior to dosing to establish baseline blood glucose.

Following dosing, samples will be taken every 5 minutes. If blood glucose is observed to fall below 2.8mmol/l subjects will be given a glucose drink.

Maximum blood volume removed for blood glucose monitoring will be 74ml per study arm

7.7 MEAL SCHEDULE

Subjects will be required to fast during the study but water will be available *ad libitum*. All subjects will receive a high carbohydrate meal prior to departure.

7.8 PHARMACOKINETIC BLOOD SAMPLING

4ml pre-dose blood samples will be taken from each subject 15 minutes before dosing

Following dosing blood samples will be taken every 30 minutes until 6 hours post-dose

Maximum blood volume removed for pharmacokinetic analysis will be 52ml per study arm

Blood samples will be centrifuged at 2000g for 10minutes and the plasma fraction removed and stored at $-20^{o}C$ for subsequent analysis by the Department of Pharmaceutical Sciences, University of Strathclyde.

7.9 CRITERIA FOR STUDY TERMINATION

The study day may be terminated before 6 hours if one of the following criteria is met:

1. A clear reduction in blood glucose has already been observed and the formulation has been observed to clear from the nasal cavity scintigraphically. Study may be terminated 30 minutes after clearance has been observed

2. No reduction in blood glucose has been observed but the formulation has been observed to clear from the nasal cavity scintigraphically. Study may be terminated 60 minutes after clearance has been observed.

3. If a blood glucose of 2.1mmol/l or less is recorded, subject will be given a bolus IV administration of 50% w/v glucose solution and monitored until glucose levels have returned. The subject will also be given a glucose drink and high carbohydrate meal when appropriate.

8 SCINTIGRAPHIC ANALYSIS

The data will be collected on computer and the nasal distribution will be determined by two independent trained personnel.

9 STUDY MATERIALS AND ACCOUNTABILITY

Supplies will be kept at room temperature within a locked cabinet with access limited to authorised personnel.

The following information will be displayed on formulation labels:

Study number
Volunteer number
Product identity/Batch Number
Activity at time of manufacture
Expiry date and time
For Clinical Trials Use Only
Keep Out Of Reach of Children
Name & address of clinical investigator

The Investigator will maintain a record of study medications including dates and batch numbers. A record will also be kept of dispensed formulations.

10 ADVERSE EVENTS

An adverse event (AE) is defined as:
"Any untoward medical occurrence in a patient or clinical investigation subject administered a pharmaceutical product which does not necessarily have to have a causal relationship with this treatment. An adverse event can therefore be any unfavourable and unintended sign (including an abnormal laboratory finding, for example), symptom, or disease temporally associated with the use of a medicinal product, whether or not considered related to the medicinal product"

The term "adverse event" may include any of the following events, which develop or increase in intensity or frequency during the course of the study, irrespective of causality, which is temporally associated with the study medication:

- any unfavourable change in medical history
- any unfavourable sign, symptom or disease;
- any clinically significant laboratory abnormality;
- any abnormality detected during physical examination

Clinically significant (CS) is defined as any abnormality that, in the judgement of a physician, impacts the health, safety or well-being of the study participant such that immediate follow-up (e.g., additional testing or treatment intervention) is warranted.

The adverse event information will include the start and stop date(s) and times of occurrence, intensity, frequency, course of action and any treatment required. These data will be transcribed to the appropriate CRF page, regardless of whether or not they are considered related to the study or the drug under investigation. "Related to the drug" means there is at least a remote possibility that the event may have been caused by the drug. An Investigator will determine whether or not the event is related to the study medication.

Adverse events will be graded as mild, moderate or severe according to the following definitions:

Mild:	**Aware of event but easily tolerable, causing no limitation of usual activities**
Moderate:	Causing some limitation of usual activities
Severe:	Causing inability to carry out usual activities

Adverse events will be elicited by use of a verbal probe to the subjects and via observation by the Investigator or his or her designee. Any subject who is removed from the study due to an adverse event will be followed until the outcome is determined.

Serious Adverse Events
A serious adverse event (SAE) is defined as any adverse event that is *both serious and unexpected*. In order to be considered a serious adverse event, it must fall into at least one of the following categories:

- death
- a life-threatening adverse drug experience that places the subject, in the view of the Investigator, at immediate risk of death from the reaction as it occurred
- initial hospitalisation, defined as in-patient admission to the hospital
- prolongation of existing inpatient hospitalisation, whether or not the adverse drug experience itself is serious
- persistent or significant disability/capacity which is defined as any substantial disruption of a person's ability to conduct normal life functions
- medical or surgical intervention that is required in order to preclude one of the outcomes described above
- congenital anomaly/birth defect

In the event of any SAE, the Investigator will determine the appropriate course of action and
follow-up on the event through its resolution.

11 RISKS

The perceived risks involved in this study relate to:

Radioactivity
Each volunteer will have received a total ionising radiation exposure of 0.4mSv calculated as follows:
^{99m}Tc DTPA 0.025mSv/MBq (total 16MBq i.e. 0.4mSv)

This is class 1 exposure (up to 0.5mSv) and is within the limits of everyday exposure in the normal population.

Insulin
Possible side effects of insulin administration include symptoms of low or high blood glucose levels e.g. sweating; dizziness; palpitation; tremor; hunger; restlessness. However, blood glucose will be monitored throughout the study period and any subject with a low blood glucose will be given glucose. All subjects will also be told

the symptoms they may experience and will be encouraged to report these to the supervising medics

In rare cases an allergic reaction to insulin may occur. Local allergy is described as redness, swelling, and itching at an injection site. Systemic allergy is less common and is characterised by a rash over the whole body, shortness of breath, wheezing, reduction in blood pressure, fast pulse, or sweating.

Blood Sampling
Total blood volume removed during the study will not exceed 544ml (includes pre-study laboratory safety evaluations).

12 STATISTICAL CONSIDERATIONS

The sample size for this pilot study was selected to provide descriptive data on the formulations and not to support rigorous statistical analyses.

13 CONFIDENTIALITY

The identity of all subjects will remain confidential and will be tracked by a unique identification number. In the event of any publication regarding this study, the identity of individuals will not be disclosed.

14 INDEMNITY

Insurance will be provided by the University of Strathclyde.

15 INVESTIGATOR RESPONSIBILITIES

The investigator will follow the ICH guidelines on "Good Clinical Practice".

16 PROTOCOL AMENDMENTS

All items in a protocol must be followed exactly. If a change is required, this must be enacted though a formal protocol amendment and receive approval from all authorities who approved the original protocol.

Appendix D

Patient Information Leaflet

STUDY TO COMPARE HOW NASAL INSERTS AND SOLUTIONS REMAIN IN THE NOSE AND TO STUDY NASAL UPTAKE OF INSULIN INTO THE BODY IN HEALTHY MALE VOLUNTEERS

Please read the following carefully:

Background

You are invited to take part in a research study. Before you decide to take part it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with friends, relatives and your GP if you wish. Ask us if there is anything that is not clear or if you would like more information. Take time to decide whether or not you wish to take part. Consumers for Ethics in Research (CERES) publish a leaflet entitled 'Medical Research and You'. This leaflet gives more information about medical research and looks at some questions you may want to ask. A copy may be obtained from CERES, PO Box 1365, London N16 OBW.

There will be eight subjects involved in this study. They will all be people who have volunteered to take part, in response to poster advertisements that we have placed. They must all satisfy certain inclusion and exclusion criteria in order to participate in this study. These study eligibility requirements relate mainly to the age, sex and medical history of the volunteers.

AIMS

This study aims to investigate a new way of delivering insulin, a hormone, via the nose. This could be used as a replacement for painful insulin injections used to treat diabetes.

The nasal inserts resemble foam ear plugs and are easily placed within the nose. The inserts gradually break down in the nose. We will compare three similar nasal inserts with a solution.

You will be given:

1. Nasal solution- this contains insulin in a very small amount (2 drops) of a water based solution
2. Nasal insert 1- this contains insulin and a low amount of a gel forming substance
3. Nasal insert 2- this contains insulin and a medium amount of a gel forming substance
4. Nasal insert 3- this contains insulin and a higher amount of a gel forming substance

The gel forming substance is routinely used in medicines and food. The inserts and solution all contain the same dose of insulin (49IU) which is a standard dose used in nasal studies.

We will be measuring:

- The spread of the medicine within the nose
- The blood levels of glucose
- The blood levels of insulin

The spread can be measured by taking pictures with a gamma camera. This piece of equipment detects and records gamma rays given off by radioisotopes which are incorporated into the inserts and solutions. A small amount of the gamma-emitting radioisotope Technetium-99m is added to the inserts and solutions. This radioisotope is used routinely in nuclear medicine clinics for patient diagnosis, in much larger doses than volunteers will receive in this study. The gamma camera does not emit X-rays or radioactivity.

Eight volunteers will take part in this study and each volunteer will participate in four study periods each lasting approximately 7 hours. It is up to you to decide whether or not to take part. If you do decide to take part you will be given this information sheet to keep and be asked to sign a consent form. If you decide to take part you are still free to withdraw at any time and without giving a reason. This will not affect the standard of care you receive. During the course of the study you will be informed immediately of any important information that that may come to light about any of the products being used. Your general practitioner will be informed of your participation in this study.

WHAT WILL HAPPEN TO ME IF I TAKE PART?

Enrolment

After you have read this volunteer information sheet, study personnel will be available to answer any questions that you may have. They will thoroughly explain the purpose and method of the study and any expected effects and potential risks to you. You will be asked to attend the Bio-Imaging Centre for a medical examination and to sign your consent at a mutually convenient time during the three weeks before the first study day. During this examination we will assess your past and present medical history, and a full physical examination will be carried out to determine your fitness to participate in the study. It is important that you tell us accurately about your full medical history and any previous problems you may have had after taking any medicines. You will be excluded from this study if the physician is not satisfied that you are fit to participate. Blood samples will be taken for testing to assess your health. These tests will include biochemistry, haematology and tests for Hepatitis B and C. The amount of blood taken will be about 20ml, which is less than a twentieth of a pint. Additionally, a urine sample will be taken for urinalysis and to test for drugs of abuse.

Study Design

This will be a non-randomised, non-blinded four-way cross-over study involving eight subjects. These terms are explained below.

Non-randomised trial:
This study is non-randomised. All volunteers will receive the same dose study medication, in the same sequence, via the same route.

Non-blinded:
This study is a non-blinded trial. You will know what study drug and dose you are taking.

Cross-over trial:
In a cross-over trial the groups each have the different treatments in turn. There may be a break between treatments so that the first drugs are cleared from your body before you start the new treatment. This is a cross-over study; all volunteers who complete the study will receive the same treatments.

WHAT DO I HAVE TO DO?

You will be expected to agree to the following:

- You must not drink any alcohol from 10pm on the evening before each study day.
- You must not eat any food, or drink any fluid except water, from 10pm on the evening before each study day.
- You must refrain from strenuous physical activity on study days(e.g. running, gym workout etc)
- You must not take any other medication within 21 days prior to study start and for the duration of the study. However should you become unwell you are encouraged to seek medical advice and follow any recommended treatment.

At each study period you will receive one form of nasal insulin. This will also contain a small amount of Technetium-99m. There are four study days, with at least 48 hours between each dose.

Study Schedule

We will ask you to arrive at the Bio-Imaging Centre at 8am in the morning of each study day. We will insert a needle into your arm to enable us to take blood samples, and attach a marker to your temple to help us take images. We will take some blood samples to determine your blood glucose level

We will then ask you sit in front of the gamma camera. The nasal solution will be administered using a disposable dropper. The nasal inserts will be inserted using a specialised device similar to a syringe.
Following dosing, images will be taken at regular intervals (approximately every 15 minutes) throughout the study day. We will also take regular blood samples (approximately every 5 minutes) during this time. The maximum amount of blood we will take from you per study day will be 126ml. The length of study day will be approximately 7 hours.

Throughout the day, you will be encouraged to report any symptoms you experience, particularly those associated with low blood glucose (listed below under risks associated with insulin). We will be measuring your blood glucose throughout the day and if your blood glucose falls below a predetermined level (just over 50% of normal fasting glucose levels) you will be given sugary drink and we will continue to monitor your blood glucose levels.

Water will be freely available throughout the study day. There is also a tv and video provided for your entertainment.

At the end of each study period you will be required to eat a meal. You will then be free to leave.

POST STUDY MEDICAL

Several days after the final study day you will be asked to return to the centre to have another physical examination, and blood and urine samples will be taken for laboratory testing. Again, the amount of blood taken will be about 20 ml.

WHAT ARE THE POSSIBLE DISADVANTAGES AND RISKS OF TAKING PART?

The risks involved relate to:

i) Insulin
Possible side effects of insulin administration include symptoms of low blood glucose levels e.g. sweating; dizziness; palpitation; tremor; hunger, restlessness. However we will be closely monitoring your blood glucose throughout the study period.

In rare cases an allergic reaction to insulin may occur. Systemic allergy is not common but is characterised by a rash over the whole body, shortness of breath, wheezing, reduction in blood pressure, fast pulse, or sweating.

ii) Radioactivity
The total radiation exposure you will receive from this study will be 0.4mSv, which is less than what you would receive if you had a hip X-ray.

iii) Blood Sampling

The total amount of blood taken from you over the entire study will be 544ml, which is just under a pint.

Taking part in the study is completely voluntary and you have the right to stop at any time without giving a reason and without affecting your future medical care. The local research ethics committee of Glasgow Royal Infirmary, which has the responsibility of assessing all applications for clinical research, has examined this study and has raised no objections from the point of view of medical ethics. During the course of the study you will be informed immediately of any important information that that may come to light about any of the products being used. Your general practitioner will be informed of your participation in this study.

WILL I BE COMPENSATED FOR TAKING PART?

For the time and inconvenience involved in participating in this study you will be paid the sum of £210, which is broken down as follows: £5 for the pre-study medical examination, £50 for each study day and £5 for the post-study medical examination. If you choose to withdraw from the study, you will be paid for the period that you have completed. If, however, having started the study, your participation is terminated by the study doctor for medical reasons, you will receive the full amount.

Studies in normal people have a very good safety record. Compensation for any injury caused by taking part in this study will be in accordance with the guidelines of the Association of the British Pharmaceutical Industry (ABPI). Broadly speaking the ABPI guidelines recommend that without legal commitment, you should be compensated without having to prove fault. This applies in cases where it is likely that such injury results from a procedure carried out in accordance with the protocol for the study. You will not be compensated where such injury results from any procedure carried out which is not in accordance with the protocol for the study. Your right at law to claim compensation for injury where you can prove negligence is not affected. Copies of these guidelines are available on request. For this study indemnity/insurance will be provided by the University of Strathclyde.

Confidentiality

It will be necessary for the researchers or possibly health authorities/drug regulatory agencies to access your medical records. Your participation in the study will be treated as confidential, that is, any personally identifiable information will be held and processed under secure conditions with access limited to appropriate staff or other authorised agents having a requirement to maintain the confidentiality of the information. You will not be referred to by name in any report of the study. Your identity will not be disclosed to any person, except for the purposes described above and in the event of a medical emergency or if required by law.

Your personal data will be processed electronically to determine the outcome of this study, and to provide it to health authorities/drug regulatory agencies.

You may be entitled under law to access your personal data and to have any justifiable corrections made. If you wish to do so, you should request this from the doctor conducting the study.

WHO IS ORGANISING AND FUNDING THE RESEARCH?

The study is part of a postgraduate student research project at the University of Strathclyde

Queries

For any further information you require about this study please contact:
Drs Bridget O'Mahony or Blythe Lindsay
Phone: (0141) 552 8791

Appendix E

Patient Consent Form

STUDY NUMBER: BC-028-01V2

Patient Identification Number: _____

CONSENT FORM

Title of project: Study to compare how nasal inserts and solutions remain in the nose and to study nasal uptake of insulin into the body in healthy male volunteers

Name of Researcher: _____

Please initial box

1. I confirm that I have read and understand the information sheet dated.....(version.....) for the above study and have had the opportunity to ask questions. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reasons, without my medical care or legal rights being affected. ☐

3. I understand that sections of any of my medical notes may be looked at by responsible individuals from regulatory authorities where it is relevant to my taking part in research. I give permission for these individuals to have access to my records. ☐

4. I agree to my General Practitioner being contacted to make known my participation ☐

5. I agree to take part in this study ☐

_____	_____	_____
Name of volunteer	Date	Signature
_____	_____	_____
Name of person taking consent (if different from researcher)	Date	Signature
_____	_____	_____
Researcher	Date	Signature

Appendix F (Abbreviations)

Abbreviations

AAC	Area above the curve
AUC	Area under the curve
BP	British Pharmacopoeia
CH	Cholate
CLSM	Confocal laser scanning microscope
C_{max}	Maximum plasma concentration
CNS	Central nervous system
CP 934P	Carbopol 934P
DMβCD	Dimethyl-β-cyclodextrin
DVS	Dynamic vapour sorption
GPR	General purpose reagent
HCl	Hydrochloric acid
HPMC	Hydroxypropylmethylcellulose
I^{125}	$Iodine^{125}$
IV	Intravenous
LHRH	Leutenising hormone releasing hormone
MC	Methyl cellulose
NaCl	Sodium chloride
NaCMC	Sodium carboxymethylcellulose
NaOH	sodium hydroxide
NHT	Nicotine hydrogen tartrate
PEG	Polyethylene glycol
PBS	Phosphate buffered saline
PVP	Polyvinylpyrrolidine
RH	Relative humidity
s.c.	subcutaneous
SD	Sodium deoxycholate
SDHTF	Sodium taurodihydrofusidate
SEM	Scanning electron microscope
SG	Sodium glycocholate
ST	Sodium taurocholate
TA	Texture Analyser
^{99m}Tc	99m-Technetium
Tg	Glass transition temperature
T_{max}	Time of maximum plasma concentration
USP	United States Pharmacopoeia

Publications

Abstracts and presentations

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