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Clearance characteristics of chitosan based formulations in the sheep nasal cavity

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Abstract

This paper describes the clearance characteristics of two bioadhesive nasal delivery systems in the form of chitosan microspheres and chitosan solution, from the nasal cavity of conscious sheep. The pattern of deposition and clearance of the nasal dosage forms were evaluated using a radioactive tracer and the non-invasive technique of gamma scintigraphy. The clearance of chitosan microsphere and solution formulations was compared with that of a control solution. The data show that the control was cleared rapidly from the sheep nasal cavity with a half-time of clearance (time taken for 50% clearance; $t_{50\%}$) of about 15 min. The bioadhesive chitosan delivery systems were cleared at a slower rate, with half-times of clearance of 43 min and 115 min, for solution and microsphere formulations respectively. From the results reported in this study it can be concluded that the chitosan delivery systems investigated had significantly reduced rates of clearance from the sheep nasal cavity, as compared to the control. Consequently, chitosan delivery systems have the ability to increase the residence time of drug formulations in the nasal cavity thereby providing the potential for improved systemic medication. The nasal clearance rates recorded in the sheep model mimic very closely the clearance rates found in a previous study using human subjects. It can also be concluded that the sheep can be considered a suitable model for in vivo nasal clearance studies of novel bioadhesive drug delivery systems.

Keywords: Gamma scintigraphy; Chitosan; Nasal drug delivery; Sheep model; Bioadhesion

1. Introduction

Much attention has been paid to the use of the nasal route for the systemic delivery of drugs that are conventionally administered by injection. The nose has many advantages as a potential site for drug delivery; being readily accessible facilitates self-medication, which may improve patient com-

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pliance compared to parenteral routes. The nasal mucosa has a relatively large absorptive surface area and is highly vascularised. Furthermore, the blood is drained directly from the nose into the systemic circulation, thus, avoiding first pass metabolism predominantly by the liver. However, many drugs, particularly polar drugs such as peptides and proteins, are not well absorbed from the nasal cavity when administered as simple solutions, with bioavailabilities in the order of 1% or less (Illum, 2000).

The major factors limiting the bioavailability of nasally administered drugs are the poor ability of polar compounds, especially the large molecular size peptides and proteins, to cross mucosal membranes; and the mucociliary clearance mechanism in the nasal cavity that rapidly removes non-bioadhesive solutions and powders from the absorption site and down the throat.

To overcome these problems and to facilitate the nasal absorption of polar molecules, two main approaches have been used; the modification of the permeability of the nasal mucosal membrane by employment of absorption enhancers, such as bile salts, surfactants, cyclodextrins, phospholipids and fatty acids (Fisher et al., 1991; Aungst, 1990; Hermens et al., 1990; Marttin et al., 1997); and the use of bioadhesive systems, both as liquid formulations and as powders, that decrease the mucociliary clearance of the drug formulation and thereby promote drug absorption (Nagai & Machida, 1985; Farraj et al., 1990; Illum et al., 1994; Critchley et al., 1994; Aspden et al., 1997).

The application of chitosan as a nasal drug delivery system to facilitate the absorption of peptide and protein drugs was first introduced by Illum (1992). In a later publication it was reported that chitosan promoted the passage of insulin and salmon calcitonin across the nasal mucosa in rat and sheep models (Illum et al., 1994). Chitosan has also been shown to facilitate the transport of vasopressin and buserelin across rat intestinal mucosa (Rentel et al., 1993; Luessen et al., 1996). The mechanism of action of chitosan can be attributed to its bioadhesive properties and its ability to transiently open tight junctions in the membrane (Artursson et al., 1994; Schipper et al., 1997; Dodane et al. 1999).

Chitosan has been shown by several research groups to be a mucoadhesive material. This characteristic is most likely due to an ionic interaction of the positively charged amino groups of the D-glucosamine units of chitosan with the negative sialic acid groups of mucin or other negatively charged groups of the mucosal membrane (Lehr et al., 1992; Fiebrig et al., 1994). The latter group showed that the interactions between chitosan–mucin were highly pH dependent with the strongest interaction at pH values where both the sialic acid units and the chitosan amino groups were well ionised. It was also shown recently, in a rat intestinal loop study by He et al. (1998), that chitosan microspheres were highly mucoadhesive, compared to a control microsphere preparation, in terms of binding to the intestinal wall.

Aspden et al. (1995, 1997) evaluated the effect of chitosan on mucociliary clearance, ex vivo, using the frog palate model and human nasal turbinate tissue. In both studies chitosan was found to transiently decrease mucociliary clearance; clearance rates returned to normal after removal of the chitosan. The effect of chitosan on the clearance of a saccharine tablet from the nasal cavity was investigated in human volunteers 1 h after chitosan administration and after 7 days repeated administration. There was no significant difference between the saccharine clearance times in the chitosan treated humans and the control (Aspden et al., 1997), demonstrating that any effect of chitosan on the mucociliary function is transient.

Recently, Soane et al. (1999) evaluated the nasal clearance characteristics of a range of formulations, including chitosan solution and chitosan microspheres, in human volunteers. It was found that the half- time of clearance of chitosan solution was almost doubled, 41 min compared with the solution control half-time of 21 min. The chitosan microspheres were found to be cleared even more slowly from the nasal cavity, with a clearance half-time of 84 min.

The importance of the sheep as an animal model in nasal drug delivery studies has increased considerably in recent years, and a range of studies using this model has been published (Lee et al., 1991; Farraj et al., 1990; Critchley et al., 1994;

Illum et al., 1994; Kublik & Muller, 1994; Illum et al., 2000). The nasal cavity of the sheep has a volume of 114 cm^3 and a surface area of 327 cm^2, compared to 19 cm^3 and 181 cm^2 respectively in man (Illum, 1996). The length of the nasal cavity is 8 cm in man and 18 cm in sheep. Furthermore, the nasal turbinates in sheep are double scroll turbinates, whereas those in man are single scroll. However, there is reasonable agreement between the calculated nasal surface areas per kg body weight in a 40 kg sheep (8.2 cm^2/kg) and in a 70 kg man (2.5 cm^2/kg) compared to other laboratory animals used in nasal delivery studies such as the dog (10 kg; 22 cm^2/kg), the monkey (7kg; 7.7 cm^2/kg) and the rat (250 g; 41.6 cm^2/kg). Moreover, it has been shown for most compounds that the nasal absorption data obtained in the sheep model closely mirrors those obtained in man (Illum, 1996), however, little is known about the clearance of formulations from the nasal cavity of sheep and whether the sheep could be used as a model for clearance in man. This is of importance for both locally acting drugs and also for drugs intended for delivery to the systemic circulation, where the absorption profile can be crucial for the clinical efficacy of the drug.

Thus, we have undertaken a study to investigate the clearance of a non-bioadhesive solution and two bioadhesive formulations (in the form of a chitosan microsphere powder and a chitosan solution) from the nasal cavity of the sheep. The formulations were labelled with a gamma emitter and nasal clearance followed using the non-invasive medical imaging technique of gamma scintigraphy.

2. Materials and methods

2.1. Materials

Chitosan glutamate (Seacure UP G210, batch no. 604-583-08) with a degree of deacetylation of 83% and a molecular weight in the region of 200 kDa, was purchased from Pronova Biomedical, Oslo, Norway. The intrinsic viscosity of the chitosan was measured to be 866 ml/g. As part of the production process, chitosan glutamate was spray dried and thereby formed into microspheres (size range 5–40 μm). By nature, chitosan glutamate is positively charged, the surface charge on the microspheres (expressed as zeta potential) in the region of +40 mV. The chitosan microspheres absorb water and dissolve with time.

Technetium-99m (Tc-99m), as sodium pertechnetate, was obtained from the Department of Medical Physics, Queen's Medical Centre, Nottingham UK. Ketamine hydrochloride (Ketalar®, 100 mg/ml injection) was obtained from Parke Davis Medical, Hampshire, UK. Ultrapure water, "Elgastat UHP" (Elga, High Wycombe, UK) was used throughout All other chemicals used were of at least analytical grade.

2.2. Preparation of formulations

The radiolabelling procedure was based upon a method previously described by Soane et al. (1999). The action of stannous chloride, a powerful reducing agent, promotes technetium-99m binding at electron donating functional groups on the chitosan polymer, such as the hydroxyl functional groups, since technetium-99m prefers ligands that are able to compensate for the high positive charge of the central atom. The stability of the radio label of the formulations was demonstrated as described in Soane et al. (1999).

2.2.1. Chitosan microsphere formulation

The technetium-99m–chitosan microspheres were produced by suspending 500 mg of the microspheres in 5 ml of a 5 mg/ml concentration of $SnCl_2.2H_2O$ (in acetone) and 200 μl sodium pertechnetate containing approximately 20 MBq of activity. The microsphere suspension was continuously stirred for 5 min, centrifuged and the microspheres washed with acetone and then dried in a fumehood overnight. A 50 mg dose of the chitosan formulation had an activity of 2 MBq at the time of administration.

2.2.2. Chitosan solution formulation

A technetium-99m-labelled chitosan solution formulation was prepared by adding 4 ml of a 5 mg/ml $SnCl_2.2H_2O$ solution (in M HCl, filtered through a sterile 0.45 μm filter) and 1 ml sodium

pertechnetate containing approximately 40 MBq of activity, to 5 ml of a 1% solution of chitosan glutamate. The solution was continuously stirred for 5 min before being placed into pre-activated dialysis tubing. The resultant chitosan solution concentration was 5 mg/ml. The solution was left to dialyse against distilled water overnight. A 0.5 ml nasal dose of the resultant chitosan solution had an activity of 2 MBq at the time of administration.

2.2.3. Control formulation

The control formulation employed in this study was a 10 ml sodium pertechnetate solution. A 0.5 ml nasal dose of the solution formulation had an activity of 2 MBq at the time of administration.

2.3. Sheep studies

2.3.1. Nasal clearance studies in sheep

The sheep has many advantages as a model for intranasal drug delivery. The sheep has a large nostril and nasal cavity, enabling accurate and reproducible administration of both liquid and powder formulations comparable to those administered to humans. Sheep can be used in the conscious state during studies, and although they are usually lightly sedated to facilitate nasal dosing, the normal mucociliary clearance mechanisms should not be affected. Furthermore, sheep have a passive and tolerant nature that allows them to be handled easily.

Nasal clearance studies in sheep were carried out in the Gamma Scintigraphy Unit (GSU) of the School of Biological Sciences, Sutton Bonington Campus, Nottingham University, UK. Two male and two female cross-bred (Suffolk $\times$ Texel) sheep weighing 44 kg (SD = 3.4 kg) at the start of the study, were used. The sheep were housed in holding pens located in the GSU (approximate temperature 20°C, humidity 50%, light cycle of 12 h light/12 h dark) and had been acclimatized to these conditions for a period of 1 week prior to the study. The animals remained housed within the facility for the duration of the study. The animals were fed a pregnant ewe meal and had free access to water. The animals were not fasted prior to the study, although food was removed for the duration of each study day (the supply of water was not affected). The clearance characteristics of the three formulations, chitosan microspheres, chitosan solution and the control formulation, were evaluated in a three-way crossover study, with a 5 day washout period between successive dose administrations.

2.3.2. Dose administration

To facilitate dose administration, and as a counter measure against the animal sneezing during nasal administration, the sheep were sedated with an intravenous injection of ketamine hydrochloride (2.25 mg/kg) administered via a jugular vein. The period of sedation was approximately 3 min.

2.3.2.1. Chitosan and control solution formulations. The chitosan and control solution formulations were administered via a syringe fitted with an umbilical cannula. The cannula was inserted approximately 7 cm into the left nostril of the sheep, and 0.5 ml solution, containing approximately 2 MBq of radioactivity, was gently instilled into the nasal cavity.

2.3.2.2. Chitosan microsphere formulation. The chitosan powder formulation was administered via a tracheal tube containing the pre-weighed dose attached to one-way bellows. A 10% excess of the powder was loaded into the tube to compensate for the powder that normally remains in the tube after administration. The tube was inserted approximately 7 cm into the left nostril prior to delivery of the powder by gentle actuation of the bellows. A nasal dose in the region of 50 mg chitosan powder with 2 MBq of radioactivity (assuming 10% losses; residual dose was not measured) was delivered nasally.

2.3.3. Gamma scintigraphy

The deposition, distribution and subsequent clearance of the various nasal formulations was evaluated by gamma scintigraphy, using a Maxi Camera II Gamma Camera (General Electric) fitted with a low energy (140 keV) collimator. The gamma camera channel was set at 141 $\pm$ 10% keV; the peak energy of technetium-99m is 141 keV.

The sheep were manually restrained in front of the gamma camera with the head of each sheep positioned about 5 cm from the collimator. Previous "dry runs" had demonstrated that the sheep could be restrained comfortably in front of the gamma camera for a period of over 60 s. The fleece on the head and neck of each animal was marked with animal stock marker which was lined-up with marks on the collimator head to ensure that each sheep was roughly in the same position when each scintigraphic image was taken. Static lateral view scintigraphic images, of 30 s duration, of the head/neck of each sheep were recorded directly after administration of the formulation (denoted as time 0) and at 5, 15, 30, 45, 60, 90, 120 and 180 min post administration. These images were stored on magnetic tape for subsequent analysis using the MICAS computer programme.

Prior to scintigraphic image analysis, the images displayed on a visual display unit (VDU) were superimposed on to an acetate sheet, using a permanent marker pen, to provide a template of the outline of the nasal cavity and nasopharynx/pharynx of each sheep. The template was used to facilitate the drawing of a region of interest (ROI) for the nasal region on each VDU image. The radioactivity (counts per cell) in the nasal ROI, corrected for both background activity and radioactive decay, was calculated. For determination of nasal clearance, the radioactivity present in the nasal ROI at zero time was used to denote the total amount of radioactivity deposited in the nasal cavity. For each subsequent image, the activity in the nasal cavity was expressed as the percentage of the total (time 0) nasal radioactivity. Thus, the clearance of each formulation from the nasal cavity was evaluated from the decrease in percentage activity against time. An estimate of the half-time of nasal clearance ($t_{50}\%$) was determined by interpolation of a plot of percentage nasal radioactivity versus time for each individual animal.

The results were analysed using one-way analysis of variance (ANOVA) by a InStat 2.03 computer programme.

The study was approved by the local Ethics Committee and performed under a Project Licence granted by the Home Office.

3. Results and discussion

The mucociliary clearance mechanism in the respiratory tract provides man with a very efficient defence against inhaled particles, bacteria and irritants reaching the lungs. These agents become stuck to the viscous mucus covering the nasal epithelium and are transported posteriorly through the nasal cavity to the pharynx, down the oesophagus and to the stomach. Mucus transport is controlled by the correlated movement of the cilia, which beat with a frequency of 1000 strokes per minute. Each stroke of a cilium consists of a rapid forward movement, where the tip of the cilium catches the mucus and transports this forward, and a slow return movement, where the cilium is bent and only moves in the surrounding pericellular fluid, thereby not affecting the movement of the viscous mucus layer. In man the mucus flow rate in the nose has been measured to be 5.3 mm/min (range 0.5–23.6 mm per minute) and hence theoretically the mucus is renewed every 10–20 minutes (Proctor & Andersen, 1976).

The clearance of inhaled materials from the nasal cavity of man has been shown to follow a biphasic pattern (Hardy et al., 1985; Aoki & Crawley, 1976; Suman et al., 1999). This biphasic pattern is the result of an initial fast rate of clearance of material from the ciliated regions of the nose, followed by a comparatively slow second phase of clearance associated with material deposited on the non-ciliated anterior region of the nose. The particle size distribution of droplets or powders administered to the nasal cavity will affect deposition in, and hence clearance from, the nasal cavity (Hardy et al., 1985; Harris et al., 1986). Furthermore, a mathematical model derived by Gonda & Gipps (1990), which includes parameters such as clearance, deposition and effect of bioadhesion, suggest that literature values were consistent with the model. It indicates that a bioadhesive formulation would remain longer in the nasal cavity, and thereby improve bioavailability and variability in absorption. However, the literature does not contain any information about the clearance of formulations from the nasal cavity of sheep. A paper by Gizurarson (1990) suggests that if one assumes a

mucociliary clearance rate of 14 mm/min in human and animal models, based on the length of the nasal cavity, the clearance half-life could be calculated to 15 min in man and 42 min in sheep. Due to lack of information in the paper concerning the method used for the calculations, it is difficult to substantiate these values. The author did not confirm the suggested half-life of clearance for the sheep model by experimental data.

Through comparison of the images recorded during this study it was possible to ascertain the approximate shape of the sheep nasal cavity and, therefore, the area of deposition after dosing. The umbilical cannula device delivered the chitosan solution and the control solution formulation to a discrete area of the anterior turbinate region (7 cm from the nostrils) in the nasal cavity. The dose subsequently spread, presumably by mucociliary clearance, over the entire nasal cavity from the anterior nasal vestibule region, right up to, and including, the nasopharynx region, within 5 min. In contrast, the tracheal tube deposited the chitosan microsphere formulation over a wider area of the anterior turbinates, but the subsequent spread was not as rapid as for the solution formulation. Accordingly, the clearance characteristics of the chitosan microsphere formulation would be expected to differ from the more widely spread chitosan solution and control solution formulations.

Examination of Fig. 1, which gives the mean activity of the various formulations within the nasal cavity ROI (%) versus time, shows that the clearance characteristics of the chitosan solution and control solution formulation did indeed exhibit biphasic-like patterns, as suggested by initial deposition pictures and the literature. In contrast, the chitosan microsphere formulation showed a more linear pattern of clearance. Hence, the clearance characteristics observed for the formulations correlated well with the observed spreading behaviour after deposition on the nasal turbinates.

The control solution formulation was cleared rapidly, with a 15 min half-time (time taken for 50% clearance), as calculated from the individual sheep clearance data (%) versus time curve (Table 1). In comparison, the bioadhesive delivery systems were retained within the nasal cavity for extended periods of time. The extent of this increased residence time was dependent upon the nature of the individual formulations. The t_{50}% for the chitosan solution formulation was 43 min, a three-fold decrease in clearance time and, for the chitosan microspheres, 115 min, an eight-fold decrease in the clearance time (Table 2). It was shown by ANOVA that the half-times ($t_{50\%}$) were significantly different ($P = 0.0018$).

The bioadhesive properties of chitosan have been described previously (Lehr et al., 1992; Henriksen et al., 1996). Such bioadhesive properties are thought to be the result of electrostatic interaction between the positively charged amino groups on the chitosan backbone and the negatively charged sialic acid groups in the mucin network. Such interactions result in the formation of adhesive complexes that increase the viscosity of the mucus and thus prolong the contact between the delivery system and the mucosa. However, before these adhesive complexes can form, intimate molecular contact must exist between the bioadhesive polymer and the mucin network. Accordingly, it is important for the chitosan microsphere formulation to absorb water from the

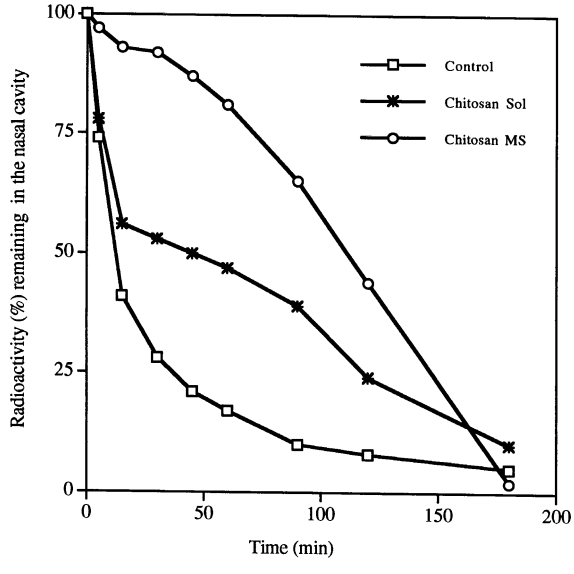

Fig. 1. The clearance characteristics of radiolabelled chitosan solution and chitosan microspheres from the sheep nasal cavity as compared to a control solution.

Table 1
The average activity remaining in the sheep nasal cavity, with standard deviations (sd), at each time point after administering chitosan and control formulations.

Time (minutes)	Mean activity (%) within the nasal cavity					
	Nasal control solution	sd (±)	Chitosan solution	sd (±)	Chitosan powder	sd (±)
0	100	0	100	0	100	0
5	74	16	78	7	97	3
15	41	18	56	5	93	4
30	28	13	53	7	92	7
45	21	9	50	8	87	9
60	17	7	47	11	81	12
90	10	5	39	6	65	41
120	8	5	24	15	44	42
180	5	3	16	14	2	1

mucin layer to establish molecular contact and thus adhesive complexes. In comparison, intimate molecular contact and thereby adhesive complex formation between the chitosan solution and the mucins should be more rapid, since the chitosan solution is already in a fully hydrated form.

The ''bioadhesive advantage'' gained by the chitosan solution through the comparatively rapid formulation of an adhesive complex, in comparison to the unhydrated chitosan microspheres, may be more than eclipsed by the lack of dehydrating/viscosity effects associated with the microsphere formulation. The consistency of the mucus layer is dependent upon the water content of the layer (Silberberg, 1990). Accordingly, it has been suggested that as the chitosan microspheres absorb water from the mucin network, the resultant dehydration produces areas of concentrated bioadhesive gel/mucus with an increased viscosity (Soane et al., 1999). These areas of increased viscosity may impart an increased resistance to the cilia beat, in comparison to the chitosan solution, since a high elasticity and low viscosity mucus layer may represent the best rheological combination for maximal clearance by the mucociliary clearance system (Puchelle et al., 1980). This would explain the differences observed between the clearance of the two chitosan formulations, with the chitosan solution having a half-time of 43 min whilst the half-time of the chitosan microsphere formulation was 115 min.

The half-time of clearance of the chitosan formulations determined in the sheep model in this study are similar to the half- times reported in a previous study in humans (Soane et al., 1999). This previous study evaluated in man, by means of gamma scintigraphy, the clearance characteristics of a range of bioadhesive polymers, including chitosan microspheres and chitosan solution formulations similar to those utilised in the present study. The time taken for 50% clearance of the control solution, chitosan solution and chitosan microsphere were 21, 41 and 84 min respectively in the human model. These values are very similar to the clearance times found in the present sheep study.

It might have been expected, on the grounds of the longer nasal cavity of the sheep (18 cm) as compared to the human nasal cavity (8 cm), that the time for 50% clearance would be longer in the sheep than in man. This was suggested by Gizurarson (1990) who calculated a t_{50}% of 15 min in man and 42 min in sheep. However, even

Table 2
The mean half-time of clearance of radiolabelled tracer from the sheep nasal cavity

Formulation	Half-time (min) (± sd)
Control	14.5 ± 8.1
Chitosan Solution	42.7 ± 30.3
Chitosan Microspheres	114.9 ± 36.6

though the nasal cavity of sheep is longer and more complex than the human nose (a double scroll turbinate in sheep compared to a single scroll turbinate in man), the structures of the epithelial membrane and the composition of the mucin network in the sheep nasal cavity are comparable to those in humans. The patterns of deposition for the two chitosan formulations were very similar in the present sheep study to those in the previous human study. Accordingly, when materials are applied directly on to the nasal mucosa of the sheep or human, the barriers that need to be overcome in order for a formulation to be retained within the nasal cavity, and for an administered drug to be absorbed into the systemic circulation, are very similar (Illum, 1996). Indeed, a comparison of compounds administered to both the sheep and human has shown very good agreement between the results obtained in sheep and human (Illum, 1996).

From the results reported in this study, it is possible to conclude that the chitosan delivery systems evaluated have good mucoadhesive properties in that they reduced the rate of clearance from the nasal cavity, compared to a simple solution control. The reduced rate of clearance provides the potential for increasing the bioavailability of drugs incorporated into these systems. It can also be concluded that the sheep is a predictive model for clearance characteristics of compounds in the human nasal cavity.

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