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In vivo evaluation of novel hyaluronan/chitosan microparticulate delivery systems for the nasal delivery of gentamicin in rabbits

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Abstract

Biodegradable microparticles containing gentamicin were prepared using chitosan hydroglutamate (CH), hyaluronic acid (HA) and a combination of both polymers by a solvent evaporation method. These formulations were administered nasally via an insufflator. Gentamicin was also administered nasally into rabbits as a solution and powder (a physical mixture of gentamicin and lactose), intravenously (IV) and intramuscularly (IM). The resultant serum levels of gentamicin were determined by Fluorescence Polarisation Immunoassay (FPIA). The bioavailability of gentamicin was poor when administered as a nasal solution (1.1%) and dry powder (2.1%) when compared with IV. However, the microparticulate systems composed of CH and HA/CH considerably enhanced the bioavailability of gentamicin (31.4 and 42.9%, respectively,) with HA microparticles inducing a less significant enhancement (23.3%). Previous in vitro dissolution and frog palate studies indicated that these microparticulate formulations were all mucoadhesive and demonstrated prolonged drug release. Such findings were translated into an increase in the bioavailability of gentamicin when compared with a simple nasal solution in vivo. When HA and CH were combined in the HA/CH formulation, the polymers appeared to improve the absorption of incorporated gentamicin synergistically in comparison to the individual polymers, suggesting a promising nasal delivery system.

Keywords: Gentamicin; Hyaluronan; Chitosan; Nasal delivery; Microparticles

1. Introduction

Although the oral route is regarded as the most convenient route of drug administration, the delivery of biopharmaceuticals via alternative routes such as the nasal, buccal, pulmonary, and rectal epithelia is becoming increasingly important. Commercially available biotechnological products that have been administered via the nasal route, for systemic absorption, include leutinising hormone releasing hormone, vasopressin and analogues, calcitonin, sumatriptan and butorphanol

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(Chien and Chang, 1985; Illum, 1999). However, difficulties are often encountered when designing effective delivery systems for such large hydrophilic drugs (Fisher et al., 1987). The development of peptide formulations is greatly limited by poor absorption as well as by difficulties with physical and chemical instability of the peptide and susceptibility to enzymatic degradation (Lee et al., 1991). To overcome this problem, various approaches have been investigated including the use of enhancer systems to increase the permeability of the mucosal barrier. Surfactants (Hirai et al., 1981), bile salts (Duchateau et al., 1986), fatty acids (Mishima et al., 1987) and various combinations of bile salts and fatty acids (Tengamnuay and Mitra, 1990) have been employed for this function. Although these systems promote the bioavailability of drugs, ciliotoxicity and damage to the nasal mucosa have been reported (Hirai et al., 1981; Tengamnuay and Mitra, 1990).

Over the last few decades, the use of mucoadhesive polymers as a non-toxic alternative to enhancer systems has been investigated. Viscous polymer solutions have been shown to increase the residence time of the drug at the nasal mucosa and thereby promote bioavailability (Morimoto et al., 1985). However, Bjork and Edman (1990) suggested that the enhanced absorption of insulin mediated by degradable starch microspheres (DSM) was not solely due to prolonged contact time. It was suggested that water uptake by DSM and subsequent swelling might cause dehydration of the epithelial cells leading to the widening of tight junctions, thereby facilitating the paracellular transport of large hydrophilic molecules. When DSM were combined with a biological enhancer, lysophosphatidylcholine (LPC), the extent of absorption was improved even further (Farraj et al., 1990). Similarly, by employing a system of starch microspheres combined with LPC, Illum et al. (1988) found that the bioavailability of gentamicin in sheep improved six-fold when compared with starch microspheres alone. More recently, both hyaluronan (HA) and chitosan have previously been reported to have excellent mucoadhesive properties (Pritchard et al., 1996) whilst chitosan has also been shown to have penetration enhancing ability (Bochard et al., 1996; Schipper et al., 1999).

Gentamicin was selected as a model drug because of its physicochemical properties (i.e. hydrophilicity), relative ease of detection and because it has been used as a model drug for intranasal studies in rats and sheep (Illum et al., 1988) and dogs (Wang and Bu, 1994). Previous in vitro studies have shown that the release of gentamicin is prolonged when formulated in HA, CH and HA/CH and that the resultant microparticles are mucoadhesive in nature (Lim et al., 2000). Consequently, the aim of this study was to investigate the effect of the novel HA/CH microparticles compared with HA and CH microparticles on the nasal absorption of a model drug, gentamicin, in vivo.

2. Materials and methods

2.1. Materials

Sodium hyaluronate (KZ 60254, molecular weight 8.5×10^5 Da), was supplied by Kiwahako (Osaka, Japan). Chitosan hydroglutamate (CH) (Protasan G210, molecular weight $1.8–2.3 \times 10^5$ Da) was donated by Pronova (Norway). Gentamicin sulphate was obtained from Fluka (Gillingham, Dorset, UK), the Innofluor gentamicin kit was obtained from Bio–Stat Diagnostic Systems, UK, New Zealand white rabbits from Harlan UK, Shaws Farm, Oxon, UK and disposable syringe filters from Fisons, Loughborough, Leicestershire, UK. All other chemicals, reagents and solvents were of analytical grade and obtained from Sigma-Aldrich Co Ltd (Poole, Dorset, UK).

2.2. Preparation of formulations

The microparticle formulations consisting of HA, CH and HA/CH were prepared by a water-in-oil (w/o) emulsification solvent evaporation technique, as described previously (Lim et al., 2000). The percentage encapsulation efficiency (calculated as the drug content per mass of microparticle) of gentamicin sulphate was found to be 46.90 ± 0.53 (HA), 28.04 ± 1.21 (HA/CH) and 13.32 ± 1.04 (CH) with a mean particle size be-

tween 19–30 μm (Lim et al., 2000). The IV formulation was prepared from a stock solution of 15 mg of gentamicin in 0.5 ml of phosphate buffer (pH 6.4). The volume administered via the right marginal ear vein was dependent upon the weight of the animal and adjusted to produce a final dose of 2 mg/kg. A similar preparation procedure was employed for intramuscular (IM) administration. For nasal administration of gentamicin solution, a stock solution of 15 mg/100 μl in phosphate buffer (pH 6.4) was prepared. Again, the final volume (a total of 87–95 μl for both nostrils) to be administered was dependent upon the weight of the animal to provide a final dose of 5 mg/kg. In all cases, not more than 50 μl of gentamicin solution (5 mg/kg) was administered into both nostrils providing a total amount of less than 100 μl. For the gentamicin–lactose mixture, equal quantities (15 mg) of gentamicin and lactose were physically mixed together. The concentration of drug in the final formulations was accurately measured using Fluorescence Polarisation Immunoassay (FPIA), as described previously (Lim et al., 2000).

2.3. Administration of nasal formulations to the rabbits

A modification of previously described devices (Ryden and Edman, 1992; Schipper et al., 1993) was developed to deliver a reproducible amount of the dry powdered formulations into the nasal cavity of the rabbits. The administration device comprised a conical tube (200 μl pipette tip, filled with the dry powder formulations) with an outlet aperture of 1 mm in diameter fitted on to a syringe filter (13 mm, 0.45 μm polypropylene housed syringe filter). This device was connected via polyethylene tubing to a 5 ml syringe to aerosolise the formulation (Fig. 1). The filter served to prevent the dry powdered formulation from entering the polyethylene tubing. The dry powder was delivered as a single bolus by pushing 5 cm^3 of air through the device where the rabbit was restrained by trained personnel and the tubing inserted about 1 to 1.5 cm into the nose before the formulation was administered.

The administration device employed to study the aerosolisation of the dry powdered formulations into the nasal cavity was validated in vitro by delivery of the dry powdered formulation under 100% relative humidity (RH) and at room humidity ($50 \pm 5\%$ RH). The 100% RH, determined by a hygrometer, was maintained using a thermostatically controlled circulating water pump. The administration device was filled with an accurately weighed amount of dry powder formulation (10–50 mg) and inserted 5 cm inside a closed chamber and aerosolised at 37 °C and 100% RH. This procedure was repeated at room temperature (25 ± 2 °C) and humidity ($50 \pm 5\%$ RH). The percentage of each dry powdered formulation delivered was calculated by Eq. (1)

$$\%\ \text{Delivered} = \frac{\text{Initial weight} - \text{residual weight}}{\text{Initial weight}} \times 100 \quad (1)$$

The amount of powdered formulation delivered under both sets of experimental conditions was determined (Table 1). The same administration device was also employed to administer gentamicin solution to the nasal cavity of the rabbit.

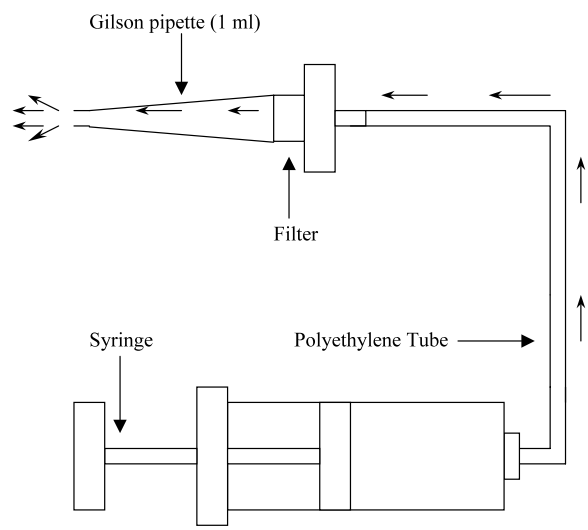

Fig. 1. Diagrammatic illustration of the device used to administer microparticles to the nasal cavity of the rabbit.

Table 1
Effect of humidity on the percentage amount of each formulation delivered by the adminstration device

Gentamicin loaded formulations (particle size (μm), mean ± S.D.)	% Delivered mean ± S.D. ($n = 4$)	% CV
HA (20.57 ± 1.33)		
Room H	99.5 ± 0.21	1.9
100% RH	85.7 ± 0.23	0.7
HA/CH (30.51 ± 2.04)		
Room H	99.3 ± 0.24	0.1
100% RH	87.5 ± 0.19	0.6
CH (30.04 ± 4.01)		
Room H	99.6 ± 0.28	0.1
100% RH	89.5 ± 0.36	0.8
Lactose/gentamicin		
Room H	94.5 ± 0.21	1.8
100% RH	83.2 ± 0.2	1.1

%CV is the percentage coefficient of variation.

2.4. In vivo studies

New Zealand white rabbits with mean weight of 2.7 ± 0.17 kg were fasted overnight and weighed before administration of the formulation. Thirty-two rabbits were randomly divided into eight groups of four. A catheter was inserted into the left marginal ear vein for blood sampling. The device described was employed to deliver the dry powdered formulations. The microparticle formulations, equivalent to approximately 6 mg of gentamicin were administered to the rabbits with one half of the dose (approximately 3 mg) being introduced to each nostril, the exact amount being adjusted according to the weight of the animals. The total weight of microparticle formulation administered as powder per nostril ranged from 5 to 15 mg. Placebo animals underwent the same procedures but were not given any gentamicin.

Blood samples (0.5 ml) were collected, at 0, 5, 10, 15, 30, 45, 60, 90, 120, 180, 240 and 360 min after drug administration. The total blood volume withdrawn during the study was 6 ml for each rabbit. The catheter was flushed with 100 μl of heparin (10 U/ml) in isotonic saline after each sample was taken. The serum was separated by centrifugation at 13 000 rpm for 5 min, collected and stored at −20 °C until assayed. The serum gentamicin concentrations were determined by FPIA.

2.5. Analysis of data

All pharmaceutical parameters (Table 2) were calculated and expressed as the mean ± S.D. All samples which showed gentamicin serum concentration below the limit of quantification (0.2 μg/ml) were set to 0 μg/ml. Estimations of the area under the curve to time t (AUC_t) and to infinity (AUC_∞) were calculated using the linear trapezoidal rule. The intranasal bioavailabilities (F) were calculated relative to the intravenous administration where IV bioavailability was considered 100% as shown in Eq. (2):

$$\%F = \frac{[AUC_{IN}][Dose(mg)]_{IVorIM}}{[AUC_{IVorIM}][Dose(mg)]_{IN}} \times 100 \quad (2)$$

Where IN represents intranasal administration, IV and IM represents intravenous and intramuscular administration, respectively. The maximum plasma concentration (C_{max}) and the time to achieve maximum concentration (t_{max}) were also obtained from each individual serum concentration curve. The half life ($t_{1/2}$) and K_e of all formulations administered were also derived and statistical analysis on the differences between AUC, AUC_∞, C_{max}, T_{max}, $t_{1/2}$ and K_e were performed using Tukey's multiple comparison test.

Table 2
Area under the curve from time 0 to 360 min (AUC_t), area under the curve from time zero to infinity (AUC_∞), and absolute bioavailability (F) following administration of gentamicin microparticulate formulations in rabbits, mean ± S.D. ($n = 4$)

Formulation	$AUC_{t=360}$ (μg.min/ml)	AUC_∞ (μg.min/ml)	F (%)
IV	560.4 ± 20.7	617.18 ± 21.5	100 ± 4.1
IM	484.4 ± 26.9	614.73 ± 24.3	99.6 ± 4.0
IN gent/soln	11.1 ± 6.4	17.31 ± 5.9	1.1 ± 0.6
HA	102.6 ± 2.6	143.56 ± 2.1	23.3 ± 1.3
CH	159.8 ± 11.8	193.64 ± 10.1	31.4 ± 2.7
HA/CH	203.9 ± 10.3	264.58 ± 9.9	42.9 ± 3.5
Gent/lact	22.0 ± 5.2	31.98 ± 5.8	2.1 ± 0.6

Gent/lact (gentamicin and lactose); IN gent/soln (Intranasal administration of gentamicin solution).

3. Results and discussion

3.1. Delivery device

The deposition of particles within the nasal cavity is highly dependent upon the device employed, hence it was essential that the device was well characterised (Mygind, 1979). As shown in Table 1, the delivery device employed shows good reproducibility with a percentage coefficient of variation of less than 2%. Due to the hygroscopic nature of the formulations, the percentage delivered was significantly higher ($P < 0.05$) at room humidity ($50 \pm 5\%$) compared with 100% RH, which was employed to simulate the conditions within the nasal cavity. However, the particle size of the microparticle had no significant ($P > 0.05$) effect on the percentage delivered, over the particle size range investigated (20–90 μm).

The site of drug deposition within the nose is dependent upon the dosage form and dose volume. Previous studies (Harris et al., 1988) have showed that the bioavailability of intranasal desmopressin from a 2×50 μl dose was 20%, which represented a marked increase over the 11% found with 1×50 μl spray or 9% obtained from the 1×100 μl intranasal dose. Such findings indicate that an optimal dosage may be obtained by administering an equal dose into each nostril. Consequently in this study, it was decided that intranasal administration of gentamicin solution would be delivered in 50 μl volumes to each nostril so that a total volume of 100 μl per rabbit was administered.

3.2. In vivo studies

Gentamicin has a high water solubility and thus it was expected to be incorporated effectively into the microparticles prepared by a w/o solvent evaporation method. Gentamicin has been employed previously in nasal absorption studies, therefore, it was thought to be an appropriate choice of drug for these investigations since comparisons could be made with the release of the active compound from other microparticles (Illum et al., 1988; Wang and Bu, 1994). Furthermore, the FPIA method employed to analyse for gentamicin has previously been well characterised (Lim et al., 2000).

In the present study, the rabbit remained conscious through the experiment, thereby providing functional mucociliary transport throughout the procedure. This is ideal for the testing of putative mucoadhesive microparticulate formulations since such formulations, if functionally successful would be required to overcome the clearance apparatus so as to provide prolonged release.

The mean serum concentration curve of a single intravenous administration of 15 mg of gentamicin over 6 h is shown in Fig. 2. The peak serum gentamicin concentration detected at the first sampling time, 5 min after administration was 7.4 ± 0.7 μg/ml and this decreased to 0.2 ± 0.1 μg/ml at the last sampling time of 6 h. The mean serum concentration curve of gentamicin following IM and IN solution is also shown in Fig. 2. Peak serum concentrations of 3.45 ± 0.4 μg/ml (IM) and 0.21 ± 0.2 μg/ml (IN) were found 30 and 15 min after administration, respectively, whereby the bioavailability of the simple nasal solution was approximately 1.1%. Such findings were in agreement with another study involving the nasal administration of gentamicin in sheep where less than 1% bioavailability was obtained when gentamicin was administered IN as a solution (Illum et al., 1988). Equally, Wang and Bu (1994) found that IN administration of gentamicin solution in dogs reached T_{max} after 15–30 min whilst Rubinstein (1983) found that IN administration of gentamicin solution in human subjects reached T_{max} after 30–60 min.

The use of powder dosage forms have long been reported to give an improved bioavailability compared with solutions, mainly due to their ability to reside longer in the nasal cavity before being cleared by the mucociliary clearance system (Illum et al., 1988). In a report by Schipper et al. (1993), two types of nasal insulin formulation were administered to rabbits, a nasal insulin solution with dimethyl-β-cyclodextrin and a nasal lactose-insulin powder mixture with dimethyl-β-cyclodextrin. The authors reported that the powder formulation exhibited 13% absolute bioavailability compared with 1.1% by the solution. In this study, a powder mixture consisting of lactose and

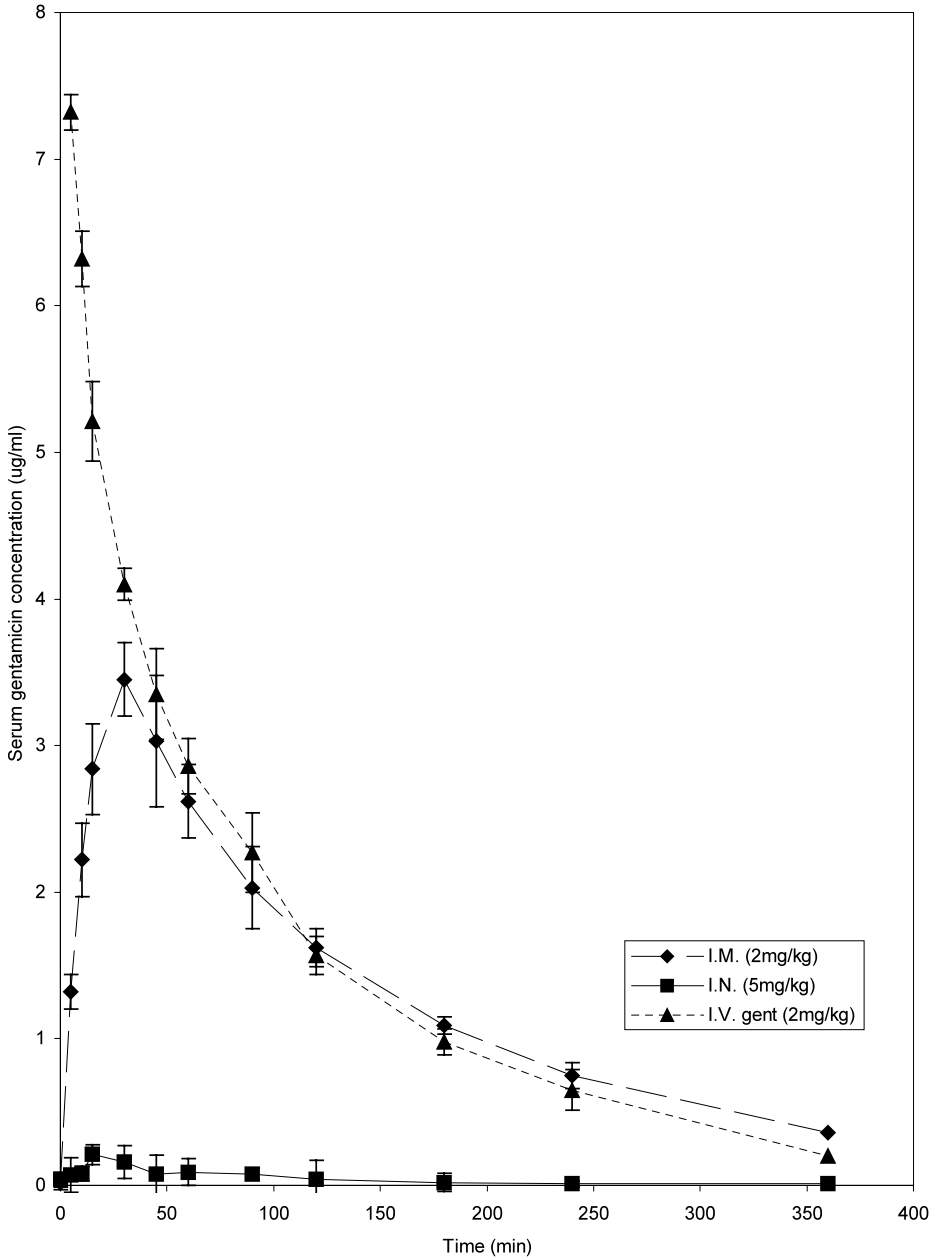

Fig. 2. Mean serum concentrations (μg/ml) of gentamicin in rabbits after intravenous (IV), intramuscular (IM) and intranasal (IN) administration of solutions, mean ± S.D. ($n = 4$).

gentamicin was employed as the control to the microparticulate formulations.

Fig. 3 compares the dry powdered formulations administered intranasally and shows that the microparticulate formulations of HA, HA/CH and CH produced sustained serum levels of gentamicin during the clearance phase compared with the gentamicin/lactose powder formulation and the

nasally administered drug solution (Fig. 2). The initial drug absorption is likely to be a consequence of the burst in release due to gentamicin loosely bound to the surface of the microparticles. A slower release would be expected to follow as a consequence of the erosion/hydration of microparticles. Such a biphasic pattern of release was also consistent with previous in vitro dissolution studies on these microparticulate systems (Lim et al., 2000).

Gentamicin administered intranasally as a dry powder formulation of gentamicin–lactose mixture in this study produced relatively low peak serum gentamicin levels (0.35 ± 0.1 μg/ml) (Fig. 3) which was nevertheless a 2-fold increase when compared with a simple IN solution (Fig. 2). Fig. 3 shows that the HA formulation gave rise to a significantly higher ($P < 0.05$) mean peak serum gentamicin level (0.61 ± 0.12 μg/ml) at 60 min which decreased below the limit of quantification after 6 h. Morimoto et al. (1991) demonstrated that the use of HA solution as a vehicle for intranasal administration increased absorption of vasopressin and a vasopressin analogue. The effect was found to correlate with the molecular weight and concentration of the polymer. In the present study, HA microparticles as a nasal delivery system for gentamicin resulted in a 2-fold increase in C_{max} of gentamicin and over 10-fold increase in bioavailability when compared with

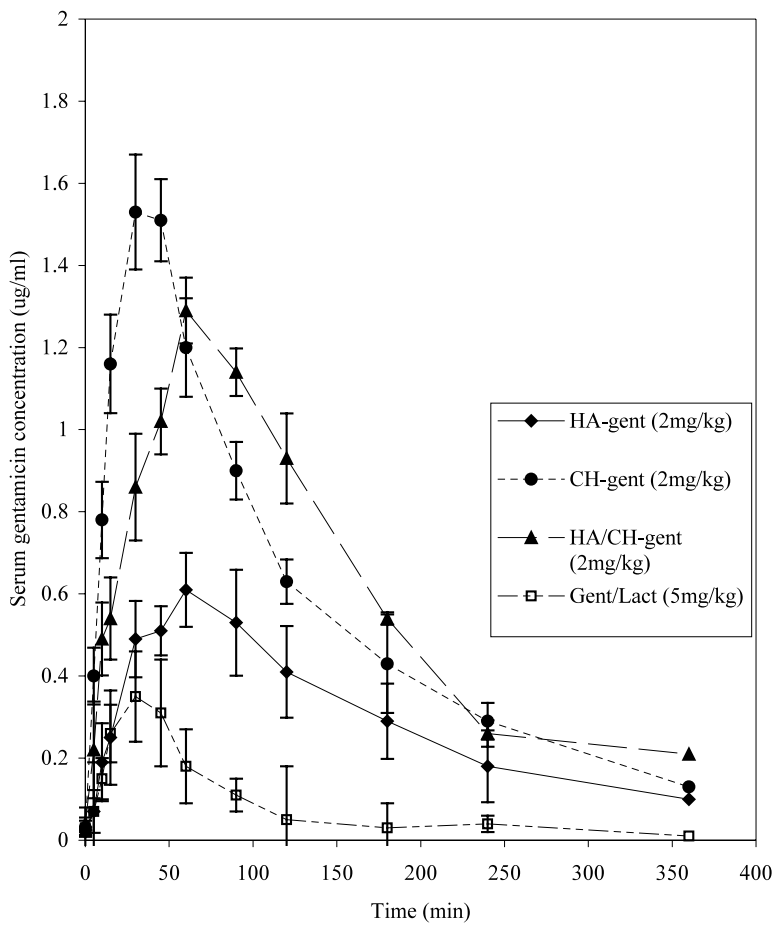

Fig. 3. Mean serum concentrations (μg/ml) of gentamicin released from microparticle formulations administered intransally, mean ± S.D. ($n = 4$).

IN gentamicin/lactose mixture. The peak plasma time from such microparticles was delayed until 60 min, reflecting the prolonged uptake of gentamicin. These observations were consistent with previous mucoadhesion frog palate studies by the authors whereby HA based formulations (HA and HA/CH microparticles) were observed to exhibit excellent mucoadhesive properties (Lim et al., 2000). In a similar study, HA ester microspheres were investigated for the nasal delivery of insulin in sheep by Illum et al. (1994). The mean bioavailability of insulin from HA ester microspheres was found to be 10%, less than half the gentamicin bioavailability achieved by the HA microparticles employed in the current study. Such marked differences are most likely to be accounted for by the larger molecular weight of insulin compared with gentamicin and the greater bioadhesive properties of the non-esterified HA employed in this study (Pritchard et al., 1996). However, direct comparisons are difficult to make, due to the differences in animal and drug model used.

The CH based formulations (HA/CH and CH microparticles), gave much higher maximum mean serum gentamicin concentrations at 30 min (1.53 ± 0.35 μg/ml) and 60 min (1.29 ± 0.34 μg/ml), respectively, (Fig. 3) than other IN test formulations. At 6 h, the mean serum concentrations were still higher than after the administration of other formulations, being 0.13 ± 0.10 μg/ml for CH and 0.21 ± 0.07 μg/ml for the HA/CH formulations. The CH and HA/CH microparticles resulted in approximately 30 and 40 times increase in gentamicin bioavailability, respectively, when compared with a simple nasal solution of gentamicin. Chitosan has been shown to enhance the absorption of hydrophilic molecules across Caco-2 cell epithelium via the paracellular transport pathway (Artursson et al., 1994), a process thought to involve the electrostatic binding of cationic chitosan with the negatively charged epithelium (Schipper et al., 1999). The CH containing microparticles used in the current study provided the highest bioavailability of intranasally administered gentamicin. In an earlier study, the nasal application of insulin with chitosan led to a significant reduction in blood glucose levels in sheep and rats (Illum et al., 1994), whilst the intraduodenal application of buserelin (MW 1299.5) and chitosan hydrochloride in a gel formulation increased the absolute bioavailability of buserelin from 0.1 ± 0.1 to 5.1 ± 1.5% (Luessen et al., 1996). Such findings provide support for the role of CH as an absorption enhancer and may explain the increased bioavailability of gentamicin from such microparticulate systems. Furthermore, the bioavailability of gentamicin in the HA/CH formulation was found to be significantly greater ($P < 0.05$) than the HA and CH microparticulate formulations alone. It is worth highlighting that the CH and HA/CH microparticles used in this study produced high absolute bioavailabilities of gentamicin in rabbits, i.e. 31 and 42%, respectively. These results suggest a synergistic effect between HA and CH in the improvement of bioavailability of gentamicin as observed for the HA/CH microparticulate formulation. The increase in bioavailability as demonstrated by the HA/CH formulation may be a result of the complementary effects of the mucoadhesive nature of HA coupled by the penetration enhancing effect of CH.

Table 3 compares the C_{max}, T_{max}, $t_{1/2}$ and K_e of all formulations. No significant difference ($P > 0.05$) was observed for the K_e of gentamicin between all formulations tested. The $t_{1/2}$ for HA based formulations were found to be not significantly different ($P > 0.05$) from the IM administration, confirming the prolonged absorption of gentamicin. However, the $t_{1/2}$ of gentamicin administered as the CH formulation was found to be markedly lower ($P < 0.001$) than that which resulted after the HA based formulations (HA and HA/CH) were given, suggesting a less prolonged release of gentamicin from the CH microparticles when compared with the HA based formulations. These findings support the results obtained from previous frog palate and dissolution studies (Lim et al., 2000).

Calvo et al. (1997) reported that the positively charged amino groups of chitosan are capable of interacting with polyanions such as sodium tripolyphosphate to form beads by inter and intramolecular linkages. Similarly, HA and CH microparticles may be based on such electrostatic

Table 3
Half life ($t_{1/2}$), elimination rate constant (K_e), maximum serum concentration (C_{max}) and time at which maximum serum concentration was achieved (T_{max}) following administration of gentamicin microparticulate formulations in rabbits, mean ± S.D. ($n = 4$)

Formulation	$t_{1/2}$ (h)	K_e (h^{-1})	C_{max} (μg/ml)	T_{max} (min)
IV	3.03 ± 0.09	0.0038 ± 0.0008	7.4 ± 0.7	5
IM	3.98 ± 0.05	0.0029 ± 0.0006	3.45 ± 0.4	30
IN gent/soln	2.9 ± 0.04	0.004 ± 0.0001	0.21 ± 0.1	15
HA	4.27 ± 0.05	0.0027 ± 0.0003	0.61 ± 0.1	60
CH	3.5 ± 0.05	0.0033 ± 0.0003	1.53 ± 0.3	30
HA/CH	3.98 ± 0.05	0.0039 ± 0.0005	1.29 ± 0.3	60
Gent/lact	3.12 ± 0.02	0.0037 ± 0.0003	0.35 ± 0.1	30
Placebo	–	–	–	–

Gent/lact (gentamicin/lactose); IN gent/soln (Intranasal administration of gentamicin solution).

interactions, however, further studies would be required to deduce if inter or intramolecular linkages are involved. The combination of the positively charged chitosan and the negatively charged HA may have resulted in a 'neutral' entity. However, based on the previous in vitro mucoadhesion studies (Lim et al., 2000) and the current in vivo data, it could be concluded that the mucoadhesive properties of HA and the penetration enhancing properties of CH has been preserved and the bioavailability of gentamicin was significantly ($P < 0.001$) greater than with either of the two polymers alone. Such a system could be tailored to increase the mucoadhesive effect or penetration enhancing effect by further optimisation of the formulation.

4. Conclusions

Characterisation of the administration device employed in this study for the delivery of dry powdered formulations proved that it was reproducible and robust. Although previous in vitro studies on gentamicin release and mucoadhesion indicated the potential of these microparticulate formulations, the results reported have demonstrated that synergistic effects in combining HA and CH to form HA/CH microparticles can be employed intranasally to obtain a high bioavailability and prolonged release of a drug.

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