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Effect of the degree of quaternisation of *N*-trimethyl chitosan chloride on absorption enhancement: in vivo evaluation in rat nasal epithelia

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Abstract

Five TMC polymers with different degrees of quaternisation (12–59%) were synthesised and administered together with [^{14}C]-mannitol in the nasal route of rats at a pH of 6.20 and 7.40, respectively. All the TMC polymers increased the nasal absorption of [^{14}C]-mannitol significantly at pH 6.20, but only TMC polymers with higher degrees of quaternisation ($>36\%$) were able to increase the absorption of this hydrophilic model compound at pH 7.40. The absorption of [^{14}C]-mannitol at pH 7.40 increased with an increase in the degree of quaternisation of TMC until a maximum absorption value was reached with TMC with a degree of quaternisation of 48%. The absorption of [^{14}C]-mannitol did not increase further, even when TMC with a higher degree of quaternisation (59%) was used. This can probably be explained by steric effects caused by the attached methyl groups and changes in the flexibility of the TMC molecules with an increase in the degree of quaternisation above an optimum value for absorption enhancement in a neutral environment. It was concluded that the degree of quaternisation of TMC plays an important role in the absorption enhancement properties of this polymer across nasal epithelia in a neutral environment.

Keywords: Nasal absorption; Absorption enhancement; Paracellular absorption; *N*-trimethyl chitosan chloride; Tight junctions

1. Introduction

Any drug has to be absorbed sufficiently from the site of administration to ensure that the maximum therapeutic effect is available to the patient. With the exception of the parenteral route all other administration routes have major physical and/or chemical barriers that could hamper drug absorption. Furthermore, the surfaces of the different routes of administration differ in their inherent permeability (Lee, 1991), enzyme activity (Lee et al., 1991) and mucus secretion (Leung et al., 1991). There is a growing interest in the nasal route for the systemic delivery of drugs that are

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active in low doses and show low oral bioavailability (Chandler et al., 1991; Verhoef and Merkus, 1994). Unlike the skin, the nasal mucosa is not constructed from highly keratinised stratum corneum, but consists of epithelial cells underlined with rich vascularity that provides direct entry of the drug into the systemic circulation via passive diffusion (Su, 1991). The respiratory epithelium of the nasal cavity consists of a single layer of mucus-covered epithelial cells. Apart from undifferentiated basal cells, mucus-producing goblet cells and ciliated as well as unciliated epithelial cells can be distinguished (Petruson et al., 1984). Similar to the gastrointestinal tract, three barriers to nasal drug absorption can be distinguished, namely, mucus and mucociliary clearance, the cellular or permeation barrier and a metabolic barrier (Mackay et al., 1991; Cornaz and Burri, 1994). Biopharmaceutical features that distinguish the nasal route from other non-parenteral routes of administration include comparatively high bioavailability, rapid kinetics of absorption comparable to intramuscular injections, avoidance of liver first-pass effect and a simple and painless mode of application (Chien, 1985).

Many hydrophilic drug candidates as well as some therapeutic peptides and macromolecules would be available for non-parenteral administration if the absorption of these compounds could be enhanced across mucosal epithelia (Artursson, 1991). The co-administration of absorption enhancing agents is one of the better-studied and attractive approaches to improve the uptake of poorly absorbable drugs. However, in some cases increased drug absorption is accompanied by toxic effects and damage to the mucosal epithelia (Schipper et al., 1996). The evaluation of absorption enhancers in human pharmacotherapy should comprise a thorough assessment of their safety including a study of the effects on the mucosal structure (Van Hoogdalem et al., 1989).

The absorption enhancing properties of chitosan recently attracted a lot of attention in pharmaceutical research. Chitosan is a cationic polymer derived from chitin, a natural polysaccharide, and it was shown by Illum et al. (1994) that chitosan is able to promote the absorption of the macromolecule, insulin, across nasal epithelia. The mechanism of action of chitosan in improving transport of drugs across mucosal membranes is thought to be a combination of the adhesion of this polymer to mucosal cells and also a transient opening of the tight junctions between adjacent epithelial cells. The interaction of chitosans with the cell membrane results in a structural reorganisation of tight junction-associated proteins, which is followed by enhanced transport through the paracellular pathway (Artursson et al., 1994; Schipper et al., 1997). However, chitosan is only effective as an absorption enhancer in acidic environments (Kotzé et al., 1999a) and precipitate from solution at neutral and basic pH values rendering it ineffective as an absorption enhancer in these environments.

It was proposed that chitosan derivatives with different physico-chemical properties, especially improved solubility at neutral and basic pH values, may have increased efficacy as absorption enhancers especially in regions such as the large and small intestine (Kotzé et al., 1999b). *N*-Trimethyl chitosan chloride (TMC), a partially quaternised derivative of chitosan with increased water solubility over a wide pH range, has been synthesised by Domard et al. (1986), Sieval et al. (1998) but its absorption enhancing effects were first studied by Kotzé et al. (1997). It was shown that the charge density of TMC, as determined by the degree of quaternisation, plays an important role on the absorption enhancing properties of this polymer especially in neutral and alkaline environments (Kotzé et al., 1999c).

The purpose of this study was to synthesise TMC polymers with different degrees of quaternisation and to evaluate its absorption enhancing properties across the nasal epithelia of rats at pH 6.20 and 7.40 to identify the optimum degree of quaternisation of TMC for nasal absorption enhancement of hydrophilic compounds in a neutral environment.

2. Method

2.1. Synthesis and characterisation of TMC polymers

TMC polymers were synthesised by a method based on the reductive methylation of chitosan

with methyl iodide in the presence of sodium hydroxide as described by Domard et al. (1986), Sieval et al. (1998). By repeating the reaction step several times under the same conditions, with the polymer obtained from each reaction step, TMC polymers with different degrees of quaternisation were synthesised (Hamman and Kotze, 2001).

The experimental conditions for the synthesis procedure include the mixing of 2 g chitosan (Pronova Biopolymer, Drammen, Norway), 4.8 g sodium iodide (Merck, South Africa), 11 ml of a 15% w/v sodium hydroxide (Merck, South Africa) solution and 11.5 ml methyl iodide (Merck, South Africa) in 80 ml *N*-methylpyrrolidone (Riedel de Haën, South Africa). This mixture was stirred on a water bath for 45 min at a temperature of 60 °C. The product was precipitated from solution with ethanol (Merck, South Africa) and isolated by centrifugation. The iodide-ion in the product was exchanged by dissolving the product in 40 ml of a 5% w/v sodium chloride (Merck, South Africa) solution and the polymer was precipitated from the solution with ethanol and isolated by centrifugation. The final product was dried in a vacuum oven at 40 °C.

Proton NMR analysis on the TMC polymers was done by a method previously described by Sieval et al. (1998). Samples (10 mg) of each synthesised polymer were dissolved in D_2O in a NMR tube and the solutions were measured in a 600 MHz DMX Brucker apparatus (Karlsruhe, Germany). The degree of quaternisation of the TMC polymers was calculated from the ^{1}H-NMR spectra of the different products with the following equation (Thanou et al., 2000):

$$\mathrm{DQ}\ (\%) = \left[\left(\int \mathrm{TM} \Big/ \int \mathrm{H}\right) \times 1/9\right] \times 100,$$

where DQ (%) is degree of quaternisation (%); $\int$ TM, integral of the trimethyl amino group (quaternary amino group) peak at 3.3 ppm; and $\int$ H, integral of the ^{1}H peaks from 4.7 to 5.7 ppm.

2.2. Preparation and nasal administration of TMC solutions

Each polymer was dissolved in 5 ml distilled water in concentrations ranging from 0.0625 to 0.5% w/v at pH 6.20 and 7.40. The pH of the final solutions was adjusted to 6.20 or 7.40 with 0.1 M HCl and 0.1 M NaOH. A volume of 10 μl [^{14}C]-mannitol (MW 182.2, specific radioactivity 57 mCi/mmol, 200 mCi/ml, Amersham Life Sciences, Little Chalfort, UK) was added to 1 ml of all the TMC solutions. Control solutions were prepared in the same way without dissolved TMC polymers.

Animal experiments were approved by the local ethical committee and were conducted according to the prescribed requirements. Healthy male Sprague–Dawley rats (Animal Research Centre at the Potchefstroom University, South Africa) with a body weight of approximately 250 g were fasted for 18 h, but water was supplied ad libitum. Six rats were used for each experiment. Anaesthesia was induced and maintained with mixtures of halothane (Fluothane®, Zebeca SA (Pty.) Ltd., Woodmead, South Africa) in medical oxygen. The apparatus for this procedure consisted of two 5 l plastic bags, containing 2 or 4% halothane in medical oxygen, each connected to one end of a three way valve. A rubber jacket fitted to the remaining end was fitted securely over the head of the rat to supply one of the two halothane mixtures as needed. The artery *carotis communis* was cannulated for blood sampling and fluid replacement. The solutions were carefully administered with a micropipette at a dose of 0.1 ml/kg body-weight (Illum et al., 1994) in the right nasal cavity of the rat. Blood samples of 300 μl (an excess of approximately 100 μl) were collected in 1.5 ml tubes at different time intervals up to 1.5 h postadministration of the solutions. Blood withdrawn were immediately replaced with an equal volume of heparinised saline at 37 °C.

2.3. Determination of [^{14}C]-mannitol plasma concentrations

A volume of 200 μl of each blood sample was prepared for scintillation counting according to

the method previously described by Faulk (1999a). In brief the method entails adding 600 μl of a mixture of quaternary ammonium hydroxide (0.5 N) in toluene (BTS-450, Beckman Coulter, USA) and isopropanol (1:2 v/v) to each blood sample and incubate them at 40 °C for 1 h. A volume of 400 μl of a 30% hydrogen peroxide were added drop wise to these blood samples while swirling them on a shaking Vortex. The samples were incubated for 15 min at ambient temperature and 30 min at 40 °C. A volume of 8 ml of a mixture of Ready Gel (Beckman Coulter, USA) and glacial acetic acid (7 ml/l) was added to the samples (Faulk, 1999b). The radioactivity present in each sample was determined with a Beckman LS 3801 liquid scintillator (Beckman Coulter, USA) and the results were corrected for dilution and expressed as [^{14}C]-mannitol concentration (% of initial dose) at the specific time point (t).

2.4. Data analysis and statistical evaluation

Averages and standard deviations were calculated for each treatment received by the rats. The maximum concentration (C_{max}) reached in the blood was obtained from the concentration–time curves and were expressed as a percentage of the total concentration of [^{14}C]-mannitol administered. The area-under-the-curve (AUC) values were calculated from the concentration–time curves using the trapezoid rule. Statistical differences between the different treatments were evaluated with the method of Tukey (studentised range distribution (HSD)) on the mean of the AUC-values. Results were considered statistically different with $P < 0.05$.

3. Results and discussion

3.1. Synthesis and characterisation of TMC polymers

The number of reaction steps used in the synthesis of the TMC polymers and the degrees of quaternisation of the products as calculated from the ^{1}H-NMR spectra are shown in Table 1. In accordance with the results in the study by Sieval et al. (1998), the degree of quaternisation of the TMC polymers increased with an increase in the number of reaction steps used in the synthesis procedure. The degree of quaternisation of TMC obtained from a one step synthesis with chitosan having a degree of deacetylation of 93% was already as high as 22.1%, and therefore, to produce TMC with a lower degree of quaternisation (12.3%), chitosan with a degree of deacetylation of 80% was used. A maximum degree of quaternisation of 59.2% for TMC was reached when four reaction steps were used in the synthesis process. Complete quaternisation of chitosan will probably be difficult because of the presence of acetyl groups on the starting polymer and possible steric effects of the attached methyl groups on adjacent quaternary amino groups. ^{1}H-NMR spectra also showed that a certain portion of the amino groups is still mono- and dimethylated and that some methylation also occurred on the three and six hydroxyl groups of chitosan.

3.2. Effect of TMC on the nasal absorption of [^{14}C]-mannitol at pH 6.20

The effect of TMC polymers (0.5% w/v) on the nasal absorption of [^{14}C]-mannitol at a pH of 6.20

Table 1
Number of reaction steps used to synthesise the different TMC polymers and the calculated degrees of quaternisation of the products

TMC polymer	Number of reaction steps	Degree of quaternisation (%)
TMC-12	1 (with 80% deacetylated chitosan)	12.3
TMC-22	1 (with 93% deacetylated chitosan)	22.1
TMC-36	2 (with 93% deacetylated chitosan)	36.3
TMC-48	3 (with 93% deacetylated chitosan)	48.0
TMC-59	4 (with 93% deacetylated chitosan)	59.2

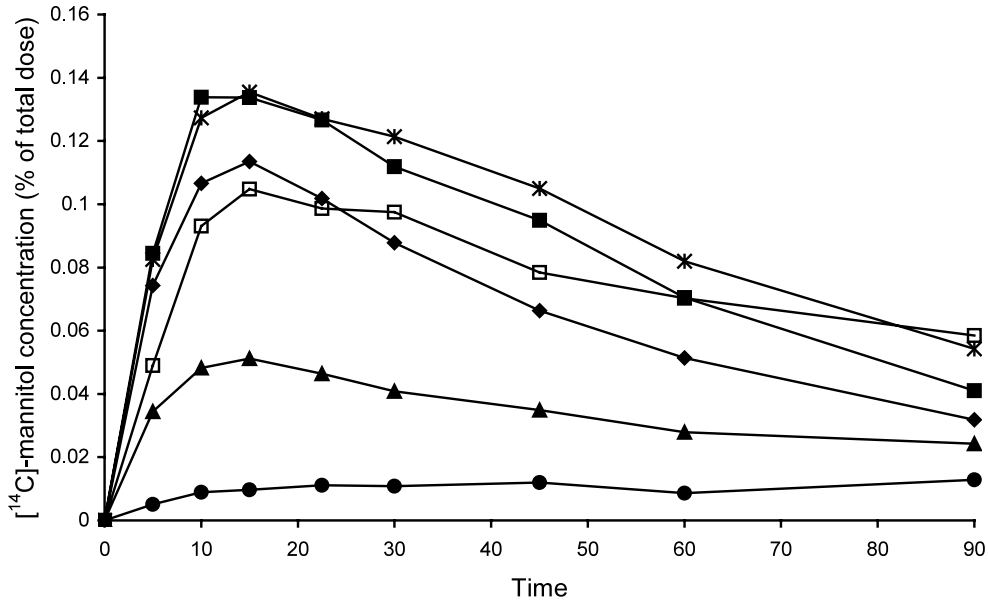

Fig. 1. Effect of TMC polymers (0.5% w/v) on the absorption of [^{14}C]-mannitol after intranasal administration in rats at a pH of 6.20. Each point represents the mean of six experiments. Control (●); TMC-12 (▲); TMC-22 (*); TMC-36 (□); TMC-48 (◆); TMC-59 (■). (Error bars omitted for reasons of clarity.)

is shown in Fig. 1. The area-under-the-curve (AUC) values calculated from the concentration–time curves with data from 0 to 90 min and the C_{max} values are given in Table 2.

A very low baseline absorption was found for [^{14}C]-mannitol in the control group at a pH of 6.20. All the TMC polymers were able to increase the nasal absorption of [^{14}C]-mannitol compared to the control group. The cumulative absorption of [^{14}C]-mannitol as a percentage of the total dose 90 min after incubation with 0.5% w/v concentrations of the polymers were: $0.31 \pm 0.17\%$ (TMC-12), $0.84 \pm 0.26\%$ (TMC-22), $0.65 \pm 0.40\%$ (TMC-36), $0.63 \pm 0.21\%$ (TMC-48), $0.80 \pm 0.44\%$ (TMC-59) and $0.08 \pm 0.05\%$ (control). These values represent a 3.9-fold (TMC-12), a 10.6-fold (TMC-22), a 8.2-fold (TMC-36), a 8.0-fold (TMC-48) and a 10.1-fold (TMC-59) increase in the cumulative percentages of [^{14}C]-mannitol absorbed.

The absorption enhancing effects of the TMC polymers in this slightly acidic environment were rapid and significant increases in the absorption of [^{14}C]-mannitol were already found 5 min postadministration, while maximum increases in the absorption were reached between 10 and 15 min postadministration. Statistically significant differences were only found between the AUC values of the control group and TMC-22, -36 and -59. The increase in absorption of [^{14}C]-mannitol could be attributed to interactions of the fixed positively charged quaternary amino groups on the C-2 position of TMC with the negatively charged anionic components of glycoproteins at the surface of the epithelial cells and within the

Table 2
AUC and C_{max} values for the concentration–time curves at pH 6.20

TMC polymer	AUC	C_{max} (% of initial dose)
Control	1.13 ± 0.44	0.01 ± 0.00
TMC-12	3.23 ± 1.59	0.05 ± 0.03
TMC-22	8.79 ± 2.82[a]	0.14 ± 0.03
TMC-36	7.24 ± 4.89[a]	0.10 ± 0.05
TMC-48	6.20 ± 2.15	0.11 ± 0.03
TMC-59	8.07 ± 4.72[a]	0.13 ± 0.06

Indicated is the mean ± S.D. of six experiments.
[a] Statistically different from control ($P < 0.05$).

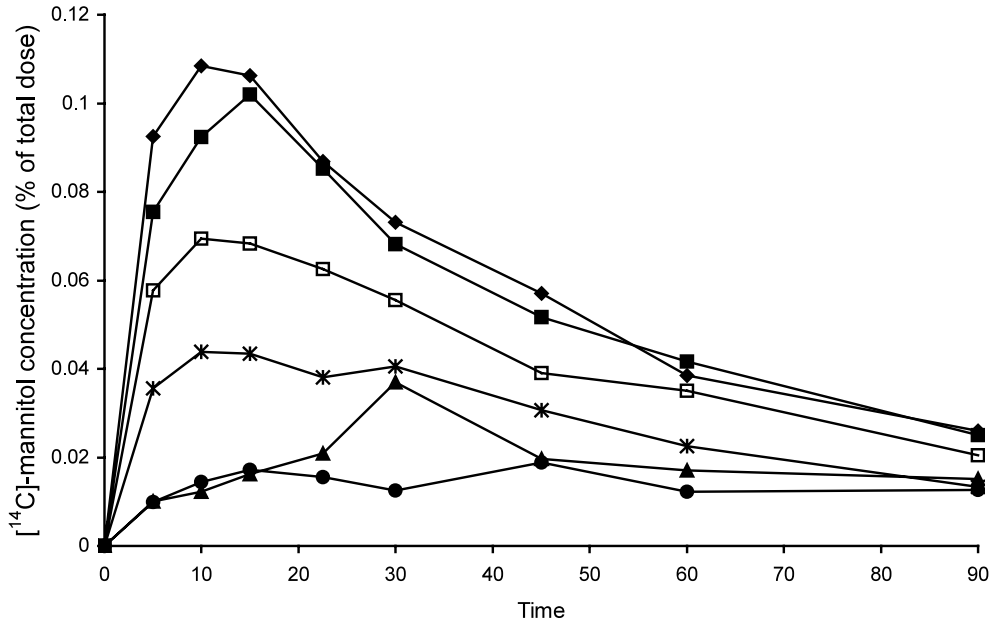

Fig. 2. Effect of TMC polymers (0.5% w/v) on the absorption of [^{14}C]-mannitol after intranasal administration in rats at a pH of 7.40. Each point represents the mean of six experiments. Control (●); TMC-12 (▲); TMC-22 (*); TMC-36 (□); TMC-48 (◆); TMC-59 (■). (Error bars omitted for reasons of clarity.)

tight junction pores of the nasal epithelia as it has been shown in a study employing monolayers of Caco-2 cells (Kotzé et al., 1997). Furthermore, mono- and dimethylated amino groups on the TMC molecules could also be protonated at pH 6.20 to form quaternary amino groups to increase the interactions with the negative charges on the epithelia and tight junctions.

3.3. Effect of TMC on the nasal absorption of [^{14}C]-mannitol at pH 7.40

The effect of TMC polymers (0.5% w/v) on the nasal absorption of [^{14}C]-mannitol at a pH of 7.40 is shown in Fig. 2. The AUC values calculated from the concentration–time curves with data from 0 to 90 min and the C_{max} values are given in Table 3.

Only a negligible amount of [^{14}C]-mannitol was absorbed across the nasal epithelia in the control group at a pH of 7.40. From Fig. 2 and Table 3 it is clear that the absorption enhancing effect of TMC depends on the degree of quaternisation of these polymers at this pH value. Only TMC polymers with higher degrees of quaternisation (TMC-36, -48 and -59) were able to increase the absorption of the hydrophilic model compound significantly compared to the control group. The cumulative absorption of [^{14}C]-mannitol as a percentage of the total dose 90 min after incubation with 0.5% w/v concentrations of the polymers were $0.15 \pm 0.06\%$ (TMC-12), $0.27 \pm 0.09\%$ (TMC-22), $0.41 \pm 0.1\%$ (TMC-36), $0.57 \pm 0.1\%$ (TMC-48), $0.54 \pm 0.09\%$ (TMC-59) and $0.11 \pm$

Table 3
AUC and C_{max} values for the concentration–time curves at pH 7.40

TMC polymer	AUC	C_{max} (% of initial dose)
Control	1.45 ± 0.78	0.02 ± 0.01
TMC-12	1.92 ± 1.01	0.04 ± 0.04
TMC-22	2.70 ± 0.85	0.04 ± 0.01
TMC-36	3.98 ± 1.11[a]	0.07 ± 0.02
TMC-48	5.37 ± 1.21[a]	0.11 ± 0.03
TMC-59	4.22 ± 0.80[a]	0.10 ± 0.02

Indicated is the mean ± S.D. of six experiments.
[a] Statistically different from control ($P < 0.05$).

0.07% (control). These values represent a 1.3-fold (TMC-12), a 2.4-fold (TMC-22), a 3.6-fold (TMC-36), a 5.0-fold (TMC-48) and a 4.8-fold (TMC-59) increase in the cumulative percentages of [^{14}C]-mannitol absorbed.

Statistical analysis showed that the increase in the absorption of [^{14}C]-mannitol differed only significantly between TMC-36, -48, -59 and the control group. This can be explained by the low charge density as determined by the relative low degree of quaternisation of TMC-12 and -22, which could not induce significant interactions with the negatively charged sites on the cell membranes and tight junctions. Furthermore, mono- and dimethylated amino groups on the TMC molecules could not be protonated at pH 7.40 to form quaternary amino groups, as was the case at pH 6.20. The TMC polymers were therefore, more effective at pH 6.20 because of the formation of a higher number of protonated, positively charged amino groups in this slightly acidic environment.

From Fig. 2 and Table 3 it is also clear that the absorption enhancing effect increased with an increase in the degree of quaternisation of TMC but reached a maximum value with TMC-48 and did not increase further, even when the degree of quaternisation was increased to 59% (no significant difference between the absorption enhancing effect of TMC-48 and -59). This can probably be explained by steric effects caused by the attached methyl groups and changes in the flexibility of the TMC molecules with an increase in the degree of quaternisation above an optimum value for absorption enhancement.

4. Conclusion

Co-administration of TMC polymers lead to enhancement of the absorption of the hydrophilic model compound, [^{14}C]-mannitol, in the nasal route of rats at both pH 6.20 and 7.40. The degree of quaternisation of TMC played an important role in the absorption enhancing properties of this polymer, especially at pH 7.40. The absorption of [^{14}C]-mannitol increased with an increase in the degree of quaternisation of TMC and only TMC polymers with higher degrees of quaternisation ($> 36\%$) were able to enhance the absorption at a pH of 7.40. The optimum degree of quaternisation for TMC was reached at 48% at pH 7.40 and no further significant increases were observed even when the degree of quaternisation was increased to 59%. It can be concluded from the results that the charge density on the TMC molecule should reach a threshold value to induce significant interactions to open the tight junctions between adjacent epithelial cells to increase paracellular transport in a neutral environment. It was explained that TMC with a higher degree of quaternisation than the optimum value for absorption enhancement in a neutral environment (pH 7.40) does not increase the absorption of the hydrophilic compound further due to possible steric effects of the attached methyl groups and changes in the flexibility of the polymer molecule.

References

Artursson, P., 1991. Cell cultures as models for drug absorption across the intestinal mucosa. Crit. Rev. Ther. Drug Carr. S. 8 (4), 305–330.

Artursson, P., Lindmark, T., Davis, S.S., Illum, L., 1994. Effect of chitosan on the permeability of intestinal epithelial cells (Caco-2). Pharm. Res. 11 (9), 1358–1361.

Chandler, S.G., Illum, L., Thomas, N.W., 1991. Nasal absorption in the rat. I: A method to demonstrate the histological effects of nasal formulations. Int. J. Pharm. 70, 19–27.

Chien, Y.W., 1985. Transdermal Systemic Medications: Fundamentals, Development Concepts and Biomedical Assessments. Elsevier, Amsterdam.

Cornaz, A.L., Burri, P., 1994. Nasal mucosal as an absorption barrier. Eur. J. Pharm. Biopharm. 40, 261–270.

Domard, A., Rinaudo, M., Terrassin, C., 1986. New method for the quaternisation of chitosan. Int. J. Biol. Macromol. 8, 105–107.

Faulk, E., 1999a. Sample preparation guide, biological fluids. Available on Internet: http://www.beckmancoulter.com/beckman/biorsrch/prodinfo/scint/sppart1.asp.

Faulk, E., 1999b. Sample preparation reducing chemiluminescence. Available on Internet: http://www.beckmancoulter.com/beckman/biorsrch/prodinfo/scint/sppart6.asp.

Hamman, J.H., Kotze, A.F., 2001. Effect of the type of base and number of reaction steps on the degree of quaternisation and molecular weight of *N*-trimethyl chitosan chloride. Drug Dev. Ind. Pharm. 27 (5), 367–374.

Illum, L., Farraj, N.F., Davis, S.S., 1994. Chitosan as a novel nasal delivery system for peptide drugs. Pharm. Res. 11 (8), 1186–1189.

Kotzé, A.F., Lueßen, H.L., De Leeuw, B.J., De Boer, (A)Bert G., Verhoef, J.C., Junginger, H.E., 1997. *N*-trimethyl chitosan chloride as a potential absorption enhancer across mucosal surfaces: in vitro evaluation in intestinal epithelial cells (Caco-2). Pharm. Res. 14 (9), 1197–1202.

Kotzé, A.F., Thanou, M.M., Lueßen, H.L., De Boer, A.G., Verhoef, J.C., Junginger, H.E., 1999a. Enhancement of paracellular drug transport with highly quaternised *N*-trimethyl chitosan chloride in neutral environments: in vitro evaluation in intestinal epithelial cells (Caco-2). J. Pharm. Sci. 88 (2), 253–257.

Kotzé, A.F., Luessen, H.L., Thanou, M., Verhoef, J.C., De Boer, A.G., Junginger, H.E., Lehr, C.-M., 1999b. Chitosan and chitosan derivatives as absorption enhancers for peptide drugs across mucosal epithelia. In: Mathiowitz, E., Chickering, D.E., Lehr, C.-M. (Eds.), Drugs and the Pharmaceutical Sciences, Bioadhesive Drug Delivery Systems, Fundamentals, Novel Approaches and Development. Marcel Dekker Inc., New York, p. 670.

Kotzé, A.F., Thanou, M.M., Lueßen, H.L., De Boer, A.G., Verhoef, J.C., Junginger, H.E., 1999c. Effect of the degree of quaternisation of *N*-trimethyl chitosan chloride on the permeability of intestinal epithelial cells (caco-2). Eur. J. Pharm. Biopharm. 47, 269–274.

Lee, V.H.L., 1991. Changing needs in drug delivery in the era of peptide and protein drugs. In: Lee, V.H.L. (Ed.), Peptide and Protein Drug Delivery. Marcell Dekker, New York, pp. 1–56.

Lee, V.H.L., Traver, R.D., Taub, M.E., 1991. Ezymatic barriers to peptide and protein drug delivery. In: Lee, V.H.L. (Ed.), Peptide and Protein Drug Delivery. Marcell Dekker, New York, pp. 303–358.

Leung, S.S., Nagai, T., Machida, Y., 1991. Mucoadhesive dosage forms for peptide and protein drug delivery. In: Lee, V.H.L. (Ed.), Peptide and Protein Drug Delivery. Marcell Dekker, New York, pp. 741–767.

Mackay, M., Williamson, I., Hastewell, J., 1991. Cell biology of epithelia. Adv. Drug Deliv. Rev. 7, 313–338.

Petruson, B., Hansson, H.A., Karlsson, G., 1984. Structural and functional aspects of cells in the nasal mucociliary system. Arch. Otolaryngol. 110, 576–581.

Schipper, N.G.M., Vårum, K.M., Artursson, P., 1996. Chitosans as absorption enhancers for poorly absorbable drugs. 1: Influence of molecular weight and degree of acetylation on drug transport across human intestinal epithelial (Caco-2) cells. Pharm. Res. 13 (11), 1686–1691.

Schipper, G.M., Olsson, S., Hoogstraate, J.A., De Boer, A.G., Vårum, K.M., Artursson, P., 1997. Chitosans as absorption enhancers for poorly absorbable drugs. 2: Mechanism of absorption enhancement. Pharm. Res. 14 (7), 923–929.

Sieval, A.B., Thanou, M., Kotzé, A.F., Verhoef, J.C., Brussee, J., Junginger, H.E., 1998. Preparation and NMR characterisation of highly substituted *N*-trimethyl chitosan chloride. Carbohydr. Polym. 36, 157–165.

Su, K.S.E., 1991. Nasal route of peptide and protein drug delivery. In: Lee, V.H.L. (Ed.), Peptide and Protein Drug Delivery. Marcell Dekker, New York, pp. 595–631.

Thanou, M., Kotzé, A.F., Scharringhausen, T., Luessen, H.L., De Boer, A.G., Verhoef, J.C., Junginger, H.E., 2000. Effect of degree of quaternisation of *N*-trimethyl chitosan chloride for enhanced transport of hydrophilic compounds across intestinal Caco-2 cell monolayers. J. Control Rel. 64, 15–25.

Van Hoogdalem, E.J., De Boer, A.G., Breimer, D.D., 1989. Intestinal drug absorption enhancement: an overview. Pharm. Ther. 44, 407–443.

Verhoef, J.C., Merkus, F.W.H.M., 1994. Nasal absorption enhancement: relevance to nasal drug delivery. In: De Boer, A.G. (Ed.), Drug Absorption Enhancement. Concepts, Possibilities, Limitations and Trends. Harwood Academic Publishers, Switzerland, pp. 119–153.