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Gelatin Microspheres as a Pulmonary Delivery System: Evaluation of Salmon Calcitonin Absorption

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Abstract

The use of negatively and positively charged gelatin microspheres for pulmonary delivery of salmon calcitonin was examined in rats. The microspheres were prepared using acidic gelatin (isoelectric point (IEP):, 5·0) and basic gelatin (IEP, 9·0) for the negatively and positively charged microspheres, respectively. The average diameters of positively charged gelatin microspheres in the dry state were 3·4, 11·2, 22·5 and 71·5 μm, and that of negatively charged gelatin microspheres was 10·9 μm. Neither positively nor negatively charged gelatin microspheres underwent any degradation in pH 7·0 PBS and there was less than 8% degradation in bronchoalveolar lavage fluid (BALF) after 8 h. In in-vitro release studies in pH 7·0 PBS, salmon calcitonin was rapidly released from positively charged gelatin microspheres within 2 h, and its cumulative release was approximately 85%. In addition, the release profiles were not influenced by particle sizes. The release rates of salmon calcitonin from negatively charged gelatin microspheres were lower than that from positively charged gelatin microspheres. The cumulative release was approximately 40% after 2 h, but there was no evidence of any sustained release.

The pulmonary absorption of salmon calcitonin from gelatin microspheres was estimated by measuring its hypocalcaemic effect in rats. The pharmacological availability after administration of salmon calcitonin in positively and negatively charged gelatin microspheres was significantly higher than that in pH 7·0 PBS. The pharmacological availability after administration of salmon calcitonin in positively charged gelatin microspheres was significantly higher than that in negatively charged gelatin microspheres. Administration of salmon calcitonin in positively charged gelatin microspheres with smaller particle sizes led to a higher pharmacological availability. The pharmacological availability after pulmonary administration of salmon calcitonin in positively charged gelatin microspheres with particle sizes of 3·4 and 11·2 μm was approximately 50%.

In conclusion, the gelatin microspheres have been shown to be a useful vehicle for pulmonary delivery of salmon calcitonin.

Pulmonary administration of peptide and protein drugs leads to a comparatively higher systemic bioavailability than that following administration by oral and other mucosal routes (Patton & Platz 1992; Patton 1996). The features of the respiratory tract favouring drug absorption include a large surface area, a thin epithelial barrier, extensive vascularization, possibly low proteolytic activity and absence of a hepatic first-pass effect. Evidence of acceptable bioavailability following pulmonary administration has been reported for a number of peptide and protein drugs such as calcitonin (Kobayashi et al 1994; Patton et al 1994), insulin (Sakr 1996), human growth hormone (Colthorpe et al 1995) and recombinant human granulocyte colony-stimulating factor (Niven et al 1994). The advantage of pulmonary administration of peptide and protein drugs could be reinforced by

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incorporating them in special formulations. Such systems would alleviate the need for multiple administrations in the case of long-term treatments. Development of formulations for pulmonary delivery of these drugs will be important for effective and safe therapy (Zeng et al 1995).

Gelatin (acidic or basic) is a denatured and biodegradable protein obtained by acidic or alkaline treatment of collagen (Tabata & Ikada 1998). This processing affects the electrical nature of collagen, yielding gelatin with a variety of isoelectric point (IEP) values. In the present study, negatively and positively charged gelatin microspheres were designed for pulmonary delivery of salmon calcitonin (chosen as a model peptide drug). The negatively and positively charged gelatin microspheres were prepared using acidic gelatin (IEP, 5·0) and basic gelatin (IEP, 9·0), respectively. Pulmonary absorption of salmon calcitonin from gelatin microspheres was estimated in rats.

Salmon calcitonin (molecular mass 3500 Da), which is a cyclic 32-amino-acid peptide, has a physiological role in the regulation of calcium homoeostasis and is a potent inhibitor of osteoclastic bone resorption (Stevenson & Evans 1981). It is effective in the treatment of Paget's disease, postmenopausal osteoporosis and malignant hypercalcaemia when given by intramuscular injection, but patients find long-term use of parenteral calcitonin very painful and this discourages good compliance.

Materials and Methods

Materials

Salmon calcitonin (24 U mg^{-1}) was obtained from Bachem Feinchemikalien AG, Switzerland. Acidic and basic gelatin (MW, 99 kDa), with IEPs of 5·0 and 9·0 respectively, was kindly supplied from Nitta Gelatin Co. Ltd, Osaka, Japan. Rhodamine iosothiocyanate (RITC) was purchased from Wako Pure Chemical Industries, Osaka, Japan. All other chemicals were of the highest purity available commercially.

Preparation of gelatin microspheres

Gelatin microspheres (diameters, 20–100 μm) were prepared by glutaraldehyde crosslinking of a gelatin aqueous solution dispersed in an oil phase in the absence of a surfactant, according to the modified method of Tabata & Ikada (1989). Briefly, 10 mL of acidic or basic gelatin aqueous solutions (10%) preheated to 40°C was added drop-wise into 375 mL of olive oil and the mixture was stirred at 420 rev min^{-1} at 40°C for 10 min to yield a water-in-oil (w/o) emulsion. Stirring was continued for 10 min at 15°C, and the microspheres were washed 3 times with acetone and isopropanol by centrifugation (3000 rev min^{-1}, 5 min, 4°C). After being air-dried, the microspheres were sized by passing them through sieves of different apertures.

Gelatin microspheres with diameters less than 20 μm were prepared using sonication to reduce the size of the w/o emulsion (Tabata et al 1999). Briefly, the mixture of 10% gelatin aqueous solution (0·2 mL) and olive oil (5 mL) was preheated and agitated with a vortex mixer at 40°C for 1 min, followed by ultrasonication at 3·0 W cm^2 for different time periods up to 40 s. The emulsion was agitated for 15 min at 15°C. Then, using the procedure described above, the resulting microspheres were washed and dried.

The non-crosslinked and dried gelatin microspheres were dispersed in 5 mL of glutaraldehyde aqueous solution (7·5 mg mL^{-1}; 25%) at 4°C for 15 h to allow crosslinking. The microspheres were further agitated in 5 mL of 10 mM glycine aqueous solution at 37°C for 1 h to block residual aldehyde groups of unreacted glutaraldehyde. The resulting microspheres were finally washed 3 times with double-distilled water by centrifugation and freeze-dried.

The water content of the prepared gelatin microspheres was calculated from their volume before and after swelling in phosphate buffered saline (pH 7·0 PBS) for 24 h at 37°C. At least 200 of both the wet and freeze-dried microspheres were examined under a light microscope to measure their diameter, then the volume of the respective microspheres was calculated. The average diameters of the positively charged gelatin microspheres in the dry state were $3{\cdot}4 \pm 0{\cdot}06$, $11{\cdot}2 \pm 0{\cdot}20$, $22{\cdot}5 \pm 0{\cdot}41$ and $71{\cdot}5 \pm 1{\cdot}59$ μm, and that of the negatively charged gelatin microspheres was $10{\cdot}9 \pm 0{\cdot}24$ μm.

For incorporation of salmon calcitonin into gelatin microspheres, 19 μL of salmon calcitonin solution in pH 7·0 PBS (0·2 mg mL^{-1}) was dropped onto 2 mg of freeze-dried gelatin microspheres, and left at 4°C for 3 h to allow the solution to penetrate the microspheres. In this study, 100% of the calcitonin aqueous solution was allowed to impregnate the microspheres during the swelling process because the solution volume was less than that which would theoretically impregnate into each microsphere. The final concentration of gelatin and salmon calcitonin in the gelatin microsphere preparation was 7 mg mL^{-1} and 15 U mL^{-1}, respectively.

In-vitro degradation of gelatin microspheres

Microspheres composed of gelatin and labelled with RITC, were prepared for the in-vitro degradation study. RITC solution (19 μL) in pH 7·0 PBS (2 mg mL^{-1}) was dropped onto 2 mg of freeze-dried gelatin microspheres, which were then left at 4°C for 3 h to allow the solution to penetrate the microspheres. The RITC-labelled gelatin microspheres were incubated in 1 mL of PBS (pH 7·0) or bronchoalveolar lavage fluid (BALF) at 37°C. BALF was prepared from a lavage of rat lungs using intratracheal instillation of PBS (pH 7·0) according to the method of Evora et al (1998). BALF (3 mL; 1·0 mg mL^{-1} protein), which was obtained by isolation of macrophages from the rat lavage fluid by centrifugation (400 *g* for 5 min at 4°C), was used in the degradation studies. At predetermined times, the dispersion was centrifuged at 400 *g* for 5 min and 0·5 mL of the supernatant was taken. The concentration of RITC in this supernatant was measured by spectrofluorometry. We confirmed that the change of RITC concentration in the supernatant accurately corresponded to the degradation of the microspheres in a series of preliminary experiments (data not shown).

In-vitro release of salmon calcitonin from gelatin microspheres

In-vitro studies of the release of salmon calcitonin from gelatin microspheres were performed by incubation in 1·5 mL of pH 7·0 PBS in a shaking water bath at 37°C. At predetermined times, 0·5 mL of the supernatant was taken and replaced with 0·5 mL of fresh pH 7·0 PBS. The released salmon calcitonin in the withdrawn supernatant was analysed using an RIA Kit (DSL-1300) from Diagnostic Systems Lab (Webster, TX).

Pulmonary administration

Male Wistar rats, 210–300 g, were fasted for 20 h before the experiment, but allowed free access to water. The rats were anaesthetized by an intraperitoneal injection of sodium pentobarbital (7·0 mg kg^{-1}), with additional doses given intraperitoneally as necessary during the experiment. Intratracheal administration of salmon calcitonin preparation was carried out according to the method of Enna & Schanker (1972).

Briefly, the trachea was exposed through a middle incision in the neck, and a 2·5-cm length of polyethylene tubing (i.d. 1·5 mm, o.d. 2·3 mm) was inserted through an incision between the fourth and fifth tracheal rings caudal to the thyroid cartilage to a depth of 0·6 cm. The salmon calcitonin gelatin microsphere preparations, suspended in pH 7·0 PBS or the salmon calcitonin solution in pH 7·0 PBS (concentration of salmon calcitonin: 15 U mL^{-1}; salmon calcitonin dose: 3·0 U kg^{-1}; dosage volume: 0·2 mL kg^{-1}), were administered intratracheally via the tube inserted in the trachea by a 100-μL glass syringe (Hamilton Co., NV). In a comparative study, salmon calcitonin was administered intravenously and intramuscularly to rats at doses of 0·5 U kg^{-1} and 1·0 U kg^{-1}, respectively. Blood samples were periodically withdrawn from the jugular vein. The absorption of salmon calcitonin was evaluated by monitoring its hypocalcaemic effect. Plasma calcium levels were determined using the Calcium C test Wako (Wako Pure Chemical Industries, Osaka, Japan). The area above the time curve of the hypocalcaemic effect (AAC) was calculated by means of trapezoidal integration. Pharmacological availability (PA) (%) was calculated as ($AAC_{i.p.}$ or $AAC_{i.m.}/D_{i.p.}$ or $D_{i.m.}$)/($AAC_{i.v.}/D_{i.v.}$) × 100, where D is the dose of salmon calcitonin.

Data analysis

All data are expressed as mean ± s.e.m. Statistical analysis was performed by Student's unpaired *t*-test. The level of significance was taken as $P < 0{\cdot}05$ and $P < 0{\cdot}01$.

Results

Degradation of gelatin microspheres and release of salmon calcitonin

The degradation of RITC-labelled gelatin microspheres was examined in pH 7·0 PBS or BALF at 37°C. Neither negatively nor positively charged gelatin microspheres (particle sizes of 10·9 μm and 11·2 μm, respectively) degraded in pH 7·0 PBS after 8 h (data not shown). Figure 1 shows the degradation profiles of negatively charged gelatin microspheres (diameter, 10·9 μm) and positively charged gelatin microspheres with various particle sizes (3·4, 11·2, 22·5 and 71·5 μm) in BALF. Less than 8% of the negatively and positively charged gelatin microspheres with various particle sizes had degraded after 8 h. There was no significant difference in the degradation of gelatin microspheres as far as charge and particle sizes were concerned. Figure 2 shows the release profiles of salmon calcitonin from negatively charged gelatin microspheres (diameter, 10·9 μm) and positively charged gelatin microspheres with various particle sizes (3·4, 11·2, 22·5 and 71·5 μm) in pH 7·0 PBS at

37°C. Salmon calcitonin was rapidly released from positively charged gelatin microspheres within 2 h; its cumulative release was approximately 85%. The release profiles were not influenced by the particle sizes. The release of salmon calcitonin from negatively charged gelatin microspheres was lower than that from positively charged gelatin microspheres, its cumulative release reaching approximately 40% after 2 h, and then levelled off.

Pulmonary absorption of salmon calcitonin from gelatin microspheres

Pulmonary absorption of salmon calcitonin from positively and negatively charged gelatin microspheres with various particle sizes were estimated in rats. Figure 3A shows the time-course of the hypocalcaemic effect in rats after pulmonary administration of salmon calcitonin ($3{\cdot}0\,U\,kg^{-1}$) in negatively and positively charged gelatin microspheres (particle sizes of 10·9 and 11·2 μm, respectively). The hypocalcaemic effect after administration of salmon calcitonin in both types of gelatin microspheres was significantly greater than that following administration in aqueous solution (in pH 7·0 PBS). The hypocalcaemic effect after administration of salmon calcitonin in positively charged gelatin microspheres was significantly greater than that after administration in negatively charged gelatin microspheres. Figure 3B shows the

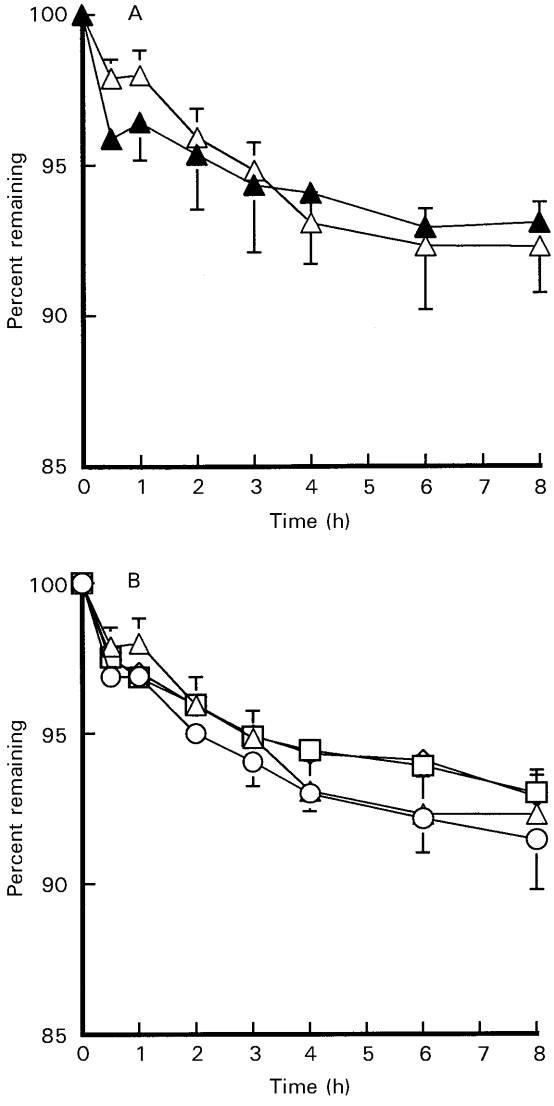

Figure 1. Degradation profiles of gelatin microspheres with different charge (A) and different particle sizes (B) in BALF at 37°C. Positively charged microspheres: 3·4 μm (○), 11·2 μm (△), 22·5 μm (□), 71·5 μm (◇). Negatively charged microspheres: 10·9 μm (▲). Each point represents the mean ± s.e.m., n = 4.

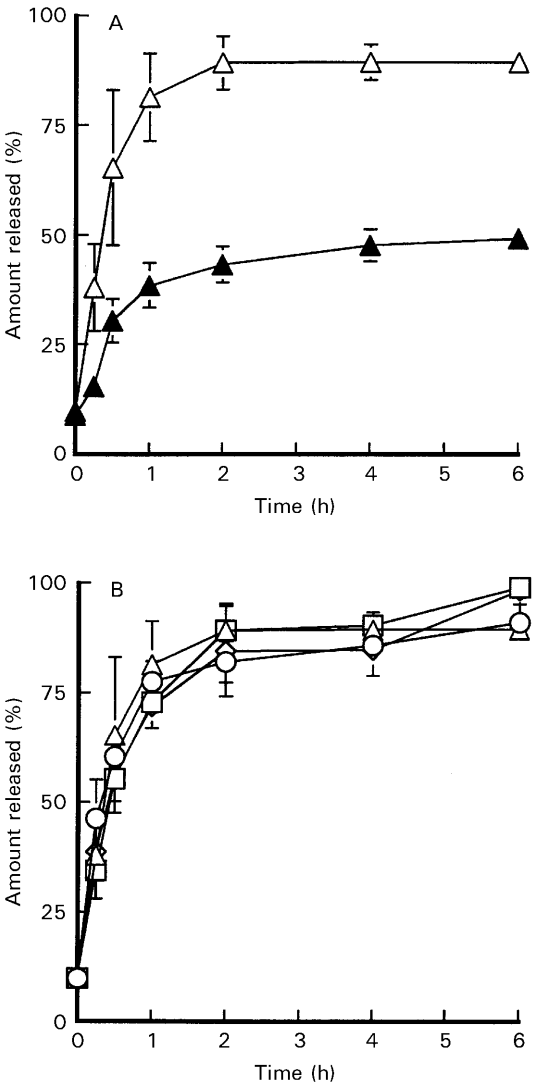

Figure 2. Release profiles of salmon calcitonin from gelatin microspheres with different charge (A) and different particle sizes (B) in pH 7·0 PBS at 37°C. Positively charged microspheres: 3·4 μm (○), 11·2 μm (△), 22·5 μm (□), 71·5 μm (◇). Negatively charged microspheres: 10·9 μm (▲). Each point represents the mean ± s.e.m., n = 4.

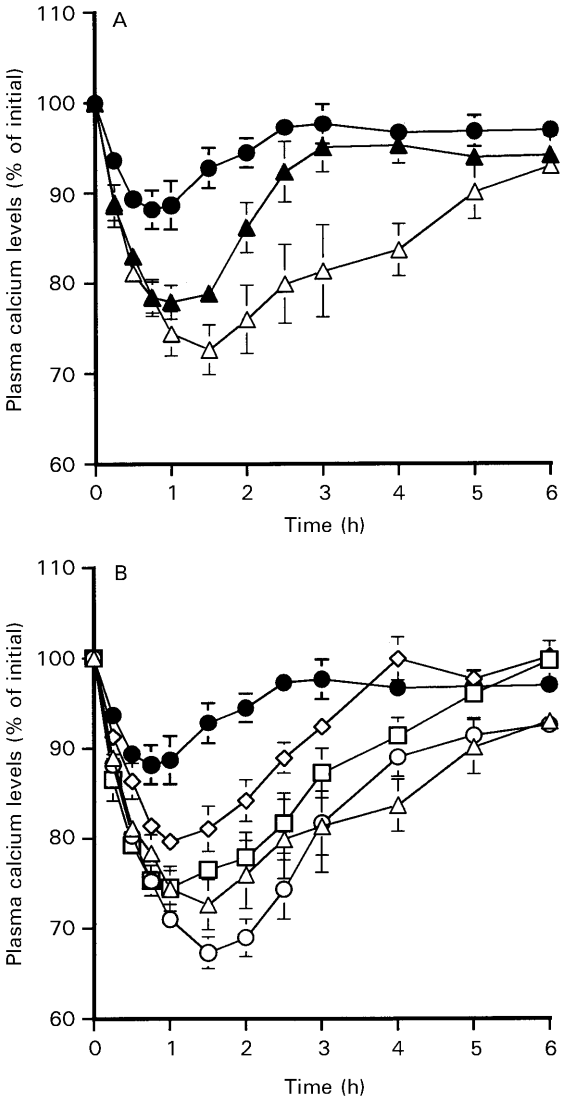

Figure 3. Time course of hypocalcaemic effect in rats after pulmonary administration of salmon calcitonin ($3{\cdot}0\,U\,kg^{-1}$) in gelatin microspheres with different charge (A) and different particle sizes (B). Solution (●); positively charged microspheres: 3·4 μm (○), 11·2 μm (△), 22·5 μm (□), 71·5 μm (◇). Negatively charged microspheres: 10·9 μm (▲). Each point represents the mean ± s.e.m. of at least four animals.

time-course of the hypocalcaemic effect in rats after pulmonary administration of salmon calcitonin ($3{\cdot}0\,U\,kg^{-1}$) in positively charged gelatin microspheres with various particle sizes (3·4, 11·2, 22·5 and 71·5 μm). Administration of salmon calcitonin in gelatin microspheres with smaller particle sizes produced a greater hypocalcaemic effect.

Comparison of the pharmacological availability of salmon calcitonin after pulmonary administration ($3{\cdot}0\,U\,kg^{-1}$) with that of intravenous administration ($0{\cdot}5\,U\,kg^{-1}$) is summarized in Table 1. These values were compared with that following intramuscular administration of salmon calcitonin solution ($1{\cdot}0\,U\,kg^{-1}$). The pharmacological availability of salmon calcitonin after pulmonary administration was approximately 15% when given in pH 7·0 PBS and was approximately 50% when given in positively charged gelatin microspheres (particle sizes: 3·4 and 11·2 μm), similar to that after intramuscular administration.

Table 1. Pharmacological availabilities after intramuscular and intraperitoneal administration of salmon calcitonin in rats.

Dosage formulations	Pharmacological availability (%)
Intramuscular administration	
Solution	49·2 ± 8·2**
Intraperitoneal administration	
Solution	14·5 ± 5·1
Negatively charged gelatin microspheres (10·9 μm)	35·4 ± 6·5**
Positively charged gelatin microspheres (3·4 μm)	51·3 ± 10·3**
Positively charged gelatin microspheres (11·2 μm)	49·2 ± 15·4**
Positively charged gelatin microspheres (22·5 μm)	38·2 ± 8·4**
Positively charged gelatin microspheres (71·2 μm)	24·7 ± 3·8*

Doses of salmon calcitonin for intravenous and intramuscular administration in solution were 0·5 and $1{\cdot}0\,U\,kg^{-1}$, respectively. The dose of salmon calcitonin for intramuscular administration in gelatin microspheres was $3{\cdot}0\,U\,kg^{-1}$. *$P < 0{\cdot}05$ and **$P < 0{\cdot}01$ vs intraperitoneal administration of salmon calcitonin solution. Each point represents the mean ± s.e.m. of at least four animals.

Discussion

The positively and negatively charged gelatin microspheres with a wide range of particle sizes (3·4–71·2 μm) were prepared using basic and acidic gelatin for pulmonary delivery of salmon calcitonin as a peptide drug. The prepared gelatin microspheres had a water content of approximately 90% (data not shown) and formed a hydrogel by swelling in aqueous solution. It is well known that gelatin hydrogels of the microsphere type are slowly degraded by enzymatic (trypsin, pepsin and collagenase) hydrolysis in the body over long periods (Tabata et al 1999). In this study, the microspheres underwent approximately 10% enzymatic degradation when incubated in BALF for 8 h. Neither the charge nor the size of the microspheres affected their degradation. Therefore enzymatic degradation may not play a major role in the elimination of microspheres from the administration site. The administered gelatin microspheres may be ultimately cleared by the ciliary movements or engulfed by the resident alveolar macrophages (Patton & Platz 1992).

Salmon calcitonin was initially released from positively and negatively charged gelatin microspheres in pH 7·0 PBS within 2 h, but there was no subsequent sustained release. The release of salmon calcitonin from negatively charged gelatin microspheres was lower than that from positively charged gelatin microspheres. Therefore, the release of salmon calcitonin depended on the charge of the microspheres. However, the release of salmon calcitonin from positively charged gelatin microspheres was not affected by the particle sizes of the microspheres. These results suggested that the release of salmon calcitonin from the microspheres used in this study occurred by the diffusion of salmon calcitonin through the microspheres, and the diffusion of salmon calcitonin in microspheres may depend on the electrostatic relationship between salmon calcitonin and microspheres. Since salmon calcitonin, which has an IEP of 8·3, is positively charged under physiological pH, the higher initial release of salmon calcitonin from positively charged gelatin (IEP, 9·0) microspheres was due to an electrostatic repulsion. However, the initial release of salmon calcitonin from negatively charged gelatin (IEP, 5·0) microspheres was suppressed by the formation of a polyion complex. In our previous report, the initial release of basic proteins with IEPs $> 7{\cdot}0$ from positively charged gelatin microspheres was very rapid, whereas acidic proteins with IEPs $< 7{\cdot}0$ were only released gradually (Tabata et al 1999). Therefore, the relationship of electrostatic forces between incorporated proteins and gelatin may play an important role as one of the factors affecting the release rate of incorporated proteins from gelatin microspheres.

The pharmacological availability of salmon calcitonin (molecular mass 3500 Da) after intratracheal administration in pH 7·0 PBS was approximately 15% and this value in rats is consistent with the results obtained by Patton et al (1994). The pharmacological availability after intratracheal administration of salmon calcitonin in positively or negatively charged gelatin microspheres was significantly greater than that after intratracheal administration of salmon calcitonin in pH 7·0 PBS. The peak hypocalcaemic effect after intratracheal administrations of salmon calcitonin in these microspheres was observed within 2 h, suggesting that the absorption-enhancing effects of microspheres occurs only during the initial period of the experiment. Furthermore, the pharmacological availability of salmon calcitonin after administration of positively charged microspheres was much greater than that with negatively charged microspheres. These results are consistent with the data on the initial release of salmon calcitonin from microspheres in-vitro. Therefore, the high concentration of salmon calcitonin at the mucosal surface of the respiratory tract, which was induced by the initial release from the microspheres, may be one of the important factors responsible for the increased salmon calcitonin absorption produced by gelatin microspheres.

The particle size is also considered to be a factor affecting the bioavailability of drugs after pulmonary administration. Brain & Valberg (1979) reported that pulmonary aerosol particles of 100 μm diameter generally do not enter the respiratory tract and are trapped in the naso/oropharynx, whereas particles < 40 μm can settle in the upper regions of the tracheobronchial tree. The alveolus of the respiratory tract favours drug absorption due to the large surface area and thin epithelial barrier (Patton 1996). It is therefore important to deliver drugs to the lower regions of the lung in order to enhance their absorption. In this study, the positively charged gelatin microspheres with smaller particle sizes appeared to reach the lower regions of rat lungs. Although the microsphere preparation was administered by intratracheal instillation in the form of a suspension, the microspheres with particle sizes of 3·4 and 10 μm may well have reached the lower regions of the lung. Then, the gelatin microspheres with smaller particle sizes exhibited a greater absorption-enhancing effect on salmon calcitonin. The fact that smaller particles can reach the lower regions of the lung may be true not only for powder formulations but also for suspension forms. Ruzinski et al (1995) reported that when 11-μm microspheres were administered by intratracheal instillation in the form of a suspension in rats, 51% and 34% of them reached the terminal bronchioles and alveolus, respectively.

We also considered that the pharmacological availability of salmon calcitonin after intratracheal administration might be attributable to enzymatic degradation in the lung mucosa. Kobayashi et al (1994) reported that the enzyme responsible for the degradation of salmon calcitonin exhibited a fourfold higher activity in the membrane fraction of lung homogenate than in the cytosol fraction. The degradation of salmon calcitonin by secreted or membrane-associated enzymes in the lung mucus layer would be physically prevented by the use of gelatin microspheres.

A number of absorption enhancers for pulmonary and other routes of mucosal administration of large hydrophilic drugs, such as peptide and protein, have been investigated during the last 10–15 years (Morita et al 1993; Marttin et al 1995). However, these absorption enhancers have not been used clinically because of concern about their safety and

toxicity. The gelatin microspheres enhanced the pulmonary absorption of salmon calcitonin in rats. Since gelatin has been extensively used in pharmaceutics and medicine in general, the safety of gelatin in the body has been established by its widespread clinical use.

In conclusion, the gelatin microspheres have been shown to be a useful vehicle for pulmonary delivery of salmon calcitonin and for increasing its absorption via the respiratory tract.

References

Brain, J. D., Valberg, P. A. (1979) Deposition of aerosol in the respiratory tract. Am. Rev. Respir. Dis. 120: 1325–1373

Colthorpe, P., Farr, S. J., Smith, I. J., Wyatt, D., Taylor, G. (1995) The influence of regional deposition on the pharmacokinetics of pulmonary-delivered human growth hormone in rabbits. Pharm. Res. 12: 356–359

Enna, S. J., Schanker, L. S. (1972) Absorption of saccharide and urea from the rat lung. Am. J. Physiol. 222: 409–414

Evora, C., Soriano, I., Rogers, R. A., Shakesheff, K. M., Hanes, J., Langer, R. (1998) Relating the phagocytosis of microparticles by alveolar macrophages to surface chemistry: the effects of 1,2-dipalmitoylphosphatidylcholine. J. Contr. Rel. S1: 143–152

Kobayashi, S., Kondo, S., Juni, K. (1994) Study on pulmonary delivery of salmon calcitonin in rats: effects of protease inhibitors and absorption enhancers. Pharm. Res. 11: 1239–1243

Marttin, E., Verhoef, J., Romeijn, S. G., Merkus, F. W. H. M. (1995) Effects of absorption enhancers on nasal epithelium in vivo: release of marker compounds in the nasal cavity. Pharm. Res. 12: 1151–1157

Morita, T., Yamamoto, A., Hashida, M., Sezaki, H. (1993) Effects of various absorption promoters on pulmonary absorption of drugs with different molecular weights. Biol. Pharm. Bull. 16: 259–262

Niven, R. W., Lott, F. D., Ip, A. Y., Cribbs, J. M. (1994) Pulmonary delivery of powders and solutions containing recombinant human granulocyte colony-stimulating factor (rhG-CSF) to the rabbit. Pharm. Res. 11: 1101–1109

Patton, J. S. (1996) Mechanisms of macromolecule absorption by the lung. Adv. Drug Deliv. Rev. 19: 3–36

Patton, J. S., Platz, R. M. (1992) Route of delivery: case studies. (2) Pulmonary delivery of peptides and proteins for systemic action. Adv. Drug Deliv. Rev. 8: 179–196

Patton, J. S., Trinchero, P., Platz, R. M. (1994) Bioavailability of pulmonary delivered peptides and proteins: α-interferon, calcitonin and parathyroid hormones. J. Contr. Rel. 28: 79–85

Ruzinski, J., Skerrett, S. J., Chi, E. Y., Martin, T. R. (1995) Deposition of particles in the lungs of infant and adult rats after direct intratracheal administration. Lab. Anim. Sci. 25: 337–340

Sakr, F. M. (1996) The pharmacokinetics of pulmonary nebulizer insulin and its effect on glucose tolerance in streptozotocin-induced diabetic rabbit. Int. J. Pharm. 128: 215–222

Stevenson, J. C., Evans, I. M. A. (1981) Pharmacology and therapeutic use of calcitonin. Drugs 21: 257–273

Tabata, Y., Ikada, Y. (1989) Synthesis of gelatin microsphere containing interferon. Pharm. Res. 6: 422–427

Tabata, Y., Ikada, Y. (1998) Protein release from gelatin matrixes. Adv. Drug Deliv. Rev. 31: 287–301

Tabata, Y., Ikada, Y., Morimoto, K., Katsumata, H., Yabuta, T., Iwanaga, K., Kakemi, K. (1999) Surfactant-free preparation of biodegradable hydrogel microspheres for protein release. J. Bioactive Compat. 14: 371–384

Zeng, X. M., Martin G. T., Marriott C. (1995) The controlled delivery of drug to the lung. Int. J. Pharm. 124: 149–164