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Research paper

The addition of calcium ions to starch/Carbopol® mixtures enhances the nasal bioavailability of insulin

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Abstract

To evaluate the influence of calcium poly(acrylates) on the nasal absorption of insulin in rabbits, starch/poly(acrylic acid) (ratio 25/75) (SD 25/75) was neutralised with NaOH and/or $Ca(OH)_2$. After neutralisation, a mixture of sodium and/or calcium carboxylate was formed depending on the $Ca(OH)_2$ concentration in the formulation. IR spectroscopy confirmed that most of the calcium molecules in the formulation interacted with acid groups of the acrylic acid polymer. Addition of $Ca(OH)_2$ to aqueous dispersions containing starch/poly(acrylic acid) yielded powders with an enhanced absorption of insulin after nasal delivery to rabbits in comparison with the equivalent powder without $Ca(OH)_2$. A mixture of SD 25/75 and $Ca(OH)_2$ at a ratio of 90/10 neutralised to pH 7.4 with NaOH induced the highest absorption of insulin, obtaining a bioavailability of ±29% (vs. 19% for an equivalent formulation without $Ca(OH)_2$). This increase in nasal delivery was possibly due to a higher elasticity after dispersing this formulation in nasal fluid and to a higher water absorbing capacity. Furthermore, after nasal delivery of (SD 25/75)/$Ca(OH)_2$ 90/10, a decrease in t_{max} was observed, possibly due to a progressive dissociation of Ca^{2+}-ions after hydration of the powder resulting in the closing of the tight junctions.

Keywords: Powder formulation; Divalent cations; Carboxylate; Nasal bioavailability; Insulin

1. Introduction

Due to their degradation in the gastro-intestinal tract by acids or proteolytic enzymes, their limited membrane permeability through the intestinal mucosa and their high first pass clearance by the liver, most peptides are not suitable for oral administration. Based on its convenience (easy access, large surface area, rich blood supply), the nasal route has been extensively investigated as an alternative route of administration [1]. However, the mucus layer, mucociliary clearance and enzymatic activity in the nasal cavity limit the nasal absorption of hydrophilic high molecular weight peptides [2]. To prevent fast clearance of the formulation from the nasal cavity, viscosity-enhancing or mucoadhesive polymers have been incorporated in the formulation in order to reduce the ciliary beat frequency and prolong residence time of the formulation into the nasal cavity [3–6]. Nagai et al. [3] incorporated insulin in powder formulations consisting of crystalline cellulose, hydroxypropyl cellulose and Carbopol® 934 which resulted in a significant decrease of plasma glucose level compared to liquid formulations after administration to rabbits and dogs. Callens et al. [6] and Pringels et al. [7] used a mixture of Amioca® starch/poly(acrylic acid) for nasal administration of insulin (1 IU insulin/mg), obtaining an absolute bioavailability of 18% in rabbits. These formulations were neutralised using NaOH to improve the viscosity-enhancing properties of the formulation. However, literature reports described that divalent ions can affect mucoadhesion of and drug release from poly(acrylic acid) polymers [8]. Tablets composed of starch-acrylic acid graft

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copolymers partially neutralised with calcium or magnesium exhibited a slower theophylline release, a better mucoadhesion and a longer adhesion time to the gingiva of dogs compared to the sodium-neutralised formulation [9]. Adding calcium or magnesium ions to a polymethylmethacrylate sodium salt used as a carrier for buccal drug delivery reduced the dissolution rate of the polymer without influencing the bioadhesive properties, thus prolonging the adhesion time in human volunteers [10].

Based on these data, the present study evaluates if the addition of calcium to a mixture of starch and poly(acrylic acid) (PAA) affected the nasal absorption of insulin in rabbits compared to mixtures formulated with starch and a sodium salt of PAA.

2. Materials and methods

2.1. Materials

Actrapid® HM 100 (100 IU/ml) (human monocomponent insulin) was obtained from Novo-Nordisk (Bagsvaerd, Denmark). The spray-dried mixture of Amioca® starch and Carbopol® 974P (ratio: 25/75, w/w) (SD 25/75) was prepared by National Starch and Chemical Company (Bridgewater, New Jersey, USA). Calcium hydroxide ($Ca(OH)_2$) and calcium carbonate ($CaCO_3$) (density: 0.30 g/ml) were obtained from Sigma–Aldrich (Bornem, Belgium) and Federa (Brussels, Belgium), respectively. All other chemicals were of analytical grade.

2.2. Preparation of insulin formulations

2.2.1. Intravenous formulation

An insulin solution of 0.8 IU/ml was prepared by diluting Actrapid® HM 100 in a sterile phosphate buffered saline solution (PBS, pH 7.4), of which 0.5 ml was administered intravenously to rabbits (3.0 ± 0.5 kg, $n = 10$).

2.2.2. Nasal powder formulations

Physical mixtures of SD 25/75 combined with $Ca(OH)_2$ or $CaCO_3$ were made in a 90/10 (w/w) ratio. To investigate the influence of the starch/Carbopol® versus $Ca(OH)_2$ ratio on the absorption of insulin, physical mixtures at a ratio of 90/1, 90/20 and 90/30 (w/w) were also prepared. The mixture SD 25/75 was used as reference.

A 500 mg powder blend was dispersed in 15 ml distilled water followed by neutralisation until pH 7.4 with 2.0 M NaOH. Then the insulin solution (Actrapid® HM 100) was added in order to obtain a final concentration of 1 IU insulin per mg powder. To obtain a powder, the aqueous dispersion was freeze-dried using an Amsco-Finn Aqua GT4 freeze-dryer (Amsco, Germany). The dispersion was frozen to 228 K within 175 min. Primary drying was performed during 13 h at 258 K and at a pressure varying between 0.8 and 1 mbar, followed by secondary drying at elevated temperature (283 K) and reduced pressure (0.1–0.2 mbar) for 7 h. After freeze-drying, the powder was sieved (63 μm) at low relative humidity (20%) and ambient temperature. The fraction below 63 μm was stored in a desiccator at 4–8 °C until use.

2.3. Nasal bioavailability study

The protocol of the animal experiments was approved by the Ethics Committee of the Institute for Agricultural and Fisheries Research (ILVO) (Merelbeke, Belgium). New Zealand white rabbits (3.0 ± 0.5 kg) were fasted 16 h prior to the experiment. Water was available ad libitum. They were sedated with an intramuscular injection of 0.05 ml/kg Placivet® (Codifar, Wommelgem, Belgium). The rabbits received 0.4 IU insulin intravenously. Ten milligrams of powder formulation (equivalent to 10 IU insulin) was administered in each nostril using polyethylene tubes (Medisize, Hillegom, The Netherlands). The powder was released from the tubes using a syringe containing 1 ml compressed air (2.5 bar). This device was based on a system developed by Sørensen [11].

The tubes were filled under conditions of low relative humidity (20%) and ambient temperature. Blood samples were collected from the ear veins at −5, 1, 5, 10, 15, 20, 30, 40, 50 and 60 min after intravenous administration and at −5, 2, 5, 10, 15, 20, 25, 30, 35, 45, 60, 90, 120, 150 and 180 min after nasal delivery of the powder formulations. The samples were centrifuged (700 g, 5 min) and the sera were frozen at −20 °C until RIA-analysis (Coat-A-Count® kit, DPC, Humbeek, Belgium). The radioactivity of the samples was quantified using a Cobra gamma counter (Canberra Packard Benelux, Zellik, Belgium). The individual serum concentration–time profiles were analysed using MW/Pharm version 3.15 (Medi-ware, Utrecht, The Netherlands) and the maximum insulin serum concentrations (C_{max}) and t_{max} values were determined from the individual serum concentration–time profiles.

The influence of the powder formulations on the absolute bioavailability, C_{max} and t_{max} of insulin was analysed using one-way ANOVA. The data and residuals were tested for normal distribution using the Kolmogorov–Smirnov test and the homogeneity of variances was tested using the Levene' s test. If the distribution of the data or residuals was not normal or the variances were not homogeneous, the data were transformed (logarithm, square root or power). Specific sets were compared using Contrast Analysis ($P < 0.05$). The software program SPSS version 11.0 was used for statistical analysis.

2.4. Physical analysis

The neutralised (SD 25/75)/$Ca(OH)_2$ powders were characterised by IR spectroscopy and X-ray diffractometry. IR spectra of the samples dispersed in KBr tablets were recorded between 400 and 4000 cm^{-1} with a resolution of 1 cm^{-1} using a Galaxy 6030 Fourier transform IR spectrophotometer (Mattson, Madison, WI, USA). X-ray powder

diffraction patterns were recorded between 2 and 60° 2θ by step-scanning with a microprocessor-controlled diffractometer system (PW 1830, Philips, Almelo, The Netherlands). Ni-filtered copper Kα radiation was used with an automatic divergence slit (PW 1836) and a graphite monochromator. The step-scanning was performed with an integration time of 4 s at intervals of 0.02° (2θ). The measurements were performed on the powder formulations without insulin.

2.5. Rheological properties

The elasticity (G′) and viscosity (G″) of the powders were determined on a TA Instruments AR 1000-N Rheometer (Zellik, Belgium) after dispersion in simulated nasal fluid (SNF) (aqueous solution containing 7.45 mg/ml NaCl, 1.29 mg/ml KCl and 0.32 mg/ml $CaCl_2$ [12]). The measurements were performed at 32 ± 0.5 °C using a cone of 4 cm with an angle of 1° and applying an oscillation stress of 1.4 Pa and a frequency of 0.1 Hz. The measurements were performed on the powder formulations without insulin.

2.6. Liquid uptake rate

The liquid uptake was studied hydrodynamically. Fifty milligrams of powder was placed on the upper side of a filter connected to a reservoir filled with SNF. The measurements were performed at 32 ± 0.5 °C. The amount of SNF absorbed was determined volumetrically as a function of time.

3. Results and discussion

3.1. Powder characterisation

To evaluate the influence of calcium poly(acrylates) on the nasal absorption of insulin, Ca^{2+}-poly(acrylates) were produced in situ by dispersing a physical mixture of spray-dried Amioca® starch/Carbopol® 974P (ratio: 25/75) and $Ca(OH)_2$ (ratios: 90/1, 90/10, 90/20 and 90/30) in distilled water. Ca^{2+}-ions effectively interacted with the carboxylic groups of PAA as less NaOH was required to neutralise (pH 7.4) the starch/PAA dispersion with increasing $Ca(OH)_2$-concentration, indicating partial neutralisation of the carboxylic acid functions of Carbopol® by $Ca(OH)_2$ (Table 1). The SD 25/75 formulation contained exclusively Na^+-carboxylate, whereas the (SD 25/75)/$Ca(OH)_2$ 90/30 formulation was mainly composed of Ca^{2+}-carboxylate.

Via X-ray diffractometry, the formation of Ca^{2+}-carboxylates could only be confirmed indirectly as the $Ca(OH)_2$-containing powders showed similar broad bands as the XRD pattern of the SD 25/75 mixture, without any of the diffraction peaks characteristic of crystalline $Ca(OH)_2$ (data are not shown).

As an intense and sharp absorption peak around 3643 cm^{-1}, which is typical for OH stretching vibration in $Ca(OH)_2$, was not detected in the IR spectra of (SD 25/75)/$Ca(OH)_2$ powder formulations, this physical analysis confirmed that most of the $Ca(OH)_2$ fraction interacted with poly(acrylic acid). All IR spectra of the SD 25/75 powder formulations had an intense and relative broad band at about 1560 cm^{-1} and a less intense absorption peak at 1454 cm^{-1} due to the asymmetric and symmetric carbonyl stretching vibration in poly(acrylate) salts [13–15] (Fig. 1). Whereas the position of the absorption peak at 1454 cm^{-1} was independent of the $Ca(OH)_2$ amount, the other band shifted with increasing $Ca(OH)_2$ concentration from 1575 cm^{-1} for a mixture without $Ca(OH)_2$

Table 1
Amount of NaOH required to adjust the pH of the dispersion to 7.4

Formulation	mg NaOH required to adjust to pH 7.4[a]
SD 25/75	437 ± 29
(SD 25/75)/$Ca(OH)_2$ 90/1	409 ± 42
(SD 25/75)/$Ca(OH)_2$ 90/10	282 ± 30
(SD 25/75)/$Ca(OH)_2$ 90/20	164 ± 30
(SD 25/75)/$Ca(OH)_2$ 90/30	34 ± 3

[a] The amount of NaOH required for neutralisation was normalised for each formulation to an equivalent amount of 1 g poly(acrylic acid) (Carbopol) ($n = 2$).

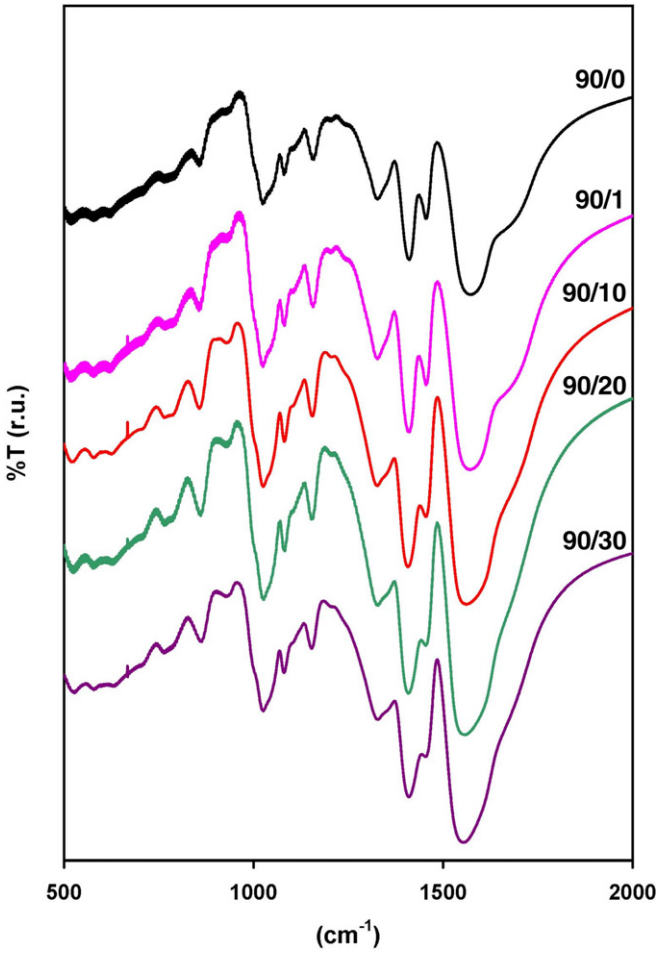

Fig. 1. IR spectra of powder formulations containing (SD 25/75)/$Ca(OH)_2$ at different ratios.

(containing only sodium poly(acrylate)) to 1552 cm^{-1} for the (SD 25/75)/Ca(OH) 90/30 formulation (containing mainly calcium poly(acrylate)). This indicated a gradual transition from an ionic structure to a chelating bidentate structure typical for calcium poly(acrylate) [14,15]. In all spectra a weak but distinct absorption at 1680 cm^{-1} was observed which appeared as a shoulder of the 1560 cm^{-1} band. This absorption is due to the carbonyl vibration in carboxyl groups, indicating that some COOH-groups of poly(acrylic acid) were not neutralised [13,14].

3.2. Nasal bioavailability

The absolute nasal bioavailability of insulin after administration of SD 25/75 mixed with $Ca(OH)_2$ at a ratio 90/10 was determined in rabbits. The addition of $Ca(OH)_2$ significantly increased the absolute nasal bioavailability ($P < 0.001$) and C_{max} ($P < 0.001$), while t_{max} significantly ($P < 0.001$) decreased (Table 2). After nasal delivery of powders containing different ratios of polymer and $Ca(OH)_2$, an increase in absorption of insulin was observed when the $Ca(OH)_2$ content increased, a maximum being reached at the 90/10 ratio. A further increase in $Ca(OH)_2$ content resulted in a sudden drop of nasal insulin absorption (Fig. 2 and Table 2). Furthermore, a linear relationship was observed between $Ca(OH)_2$ concentration and t_{max} ($P < 0.001$, Linear Contrast Analysis), this parameter decreasing at higher calcium concentrations. The type of Ca-salt used in the formulation had no effect on insulin absorption as a similar ($P > 0.05$) absolute bioavailability ($26.1 \pm 9.8\%$), C_{max} (1288 ± 670 μIU/ml) and t_{max} (28.7 ± 4.6 min) were obtained using a powder formulation with calcium carbonate (ratio 90/10).

It is known that poly(acrylates) have the potential to bind calcium ions. They were able to prevent denaturation of proteins by enzymes that are thermodynamically stabilised by calcium (e.g. trypsin or α-chymotrypsin), through deprivation of the enzyme-bound calcium [16,17]. It has also been demonstrated that poly(acrylates) are able to deplete extracellular Ca^{2+} whereby the paracellular permeability through the epithelium is increased by opening the tight junctions [18]. However, Ca^{2+}-poly(acrylates) exhibit different characteristics compared to Na^{+}-poly(acrylates). Complexation of calcium by poly(acrylic acid) derivatives decreased the particle size of dispersed swollen

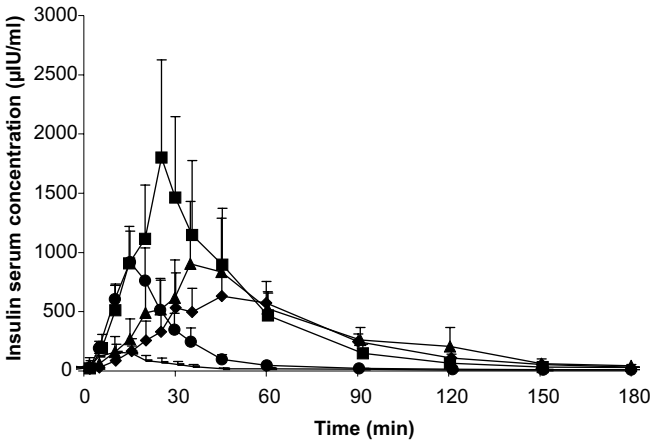

Fig. 2. Insulin serum concentration–time profiles in rabbits after nasal delivery of powder formulations (1 IU insulin/mg) containing SD 25/75 mixed with $Ca(OH)_2$ at different ratios: 90/1 (▲), 90/10 (■), 90/20 (●) and 90/30 (–). The formulation based on SD 25/75 without $Ca(OH)_2$ (♦) was used as reference.

particles of these polymers as calcium acts as a cross-linking agent between polymer chains [19]. Madsen and Peppas [20] demonstrated that calcium significantly decreased the swelling ratio of gels containing grafted copolymer networks of poly(metacrylic acid-g-ethylene glycol) compared to the swelling ratio obtained in buffers containing NaCl. This phenomenon is pH-dependent: in acid media (below pH 4.4), neither monovalent nor divalent cations affect the swelling ratio as the protonated carboxylic acid groups are not available for sodium or calcium binding. At increasing pH, the acid groups are ionised whereby sodium or calcium ions can interact with the polymer. Sodium ions (monovalent cations) can move freely in the poly(acrylate) network, whereas calcium ions (divalent cations) promote aggregation of polymer chains by intermolecular bridge formation [21]. The electrostatic repulsion between the negatively charged carboxylic acid groups is lower for calcium poly(acrylates), resulting in a decrease of the swelling force and consequently a much lower equilibrium swelling ratio [20]. These interactions between mono- and divalent ions and poly(acrylic acid) explained the decrease in t_{max} at higher $Ca(OH)_2$ content: at ratios of 90/20 and 90/30 a weaker gel was formed after dispersion in simulated nasal fluid compared to the formulation without $Ca(OH)_2$ (Table 3). The formulation at a ratio of 90/30 was even not able to form a homogeneous hydrated

Table 2
Absolute bioavailability, C_{max} and t_{max} (mean ± SD) in rabbits after nasal delivery of powders (1 IU insulin/mg) containing SD 25/75 mixed with $Ca(OH)_2$ at different ratios

Formulation	BA (%)	C_{max} (μIU/ml)	t_{max} (min)	n
[b]SD 25/75	19.2 ± 5.3	681 ± 247	50.9 ± 7.4	8
[b](SD 25/75)/$Ca(OH)_2$ 90/1	24.6 ± 9.2	958 ± 484	44.5 ± 9.6	6
[a](SD 25/75)/$Ca(OH)_2$ 90/10	29.0 ± 11.4***	1813 ± 828***	27.3 ± 3.4	8
[b](SD 25/75)/$Ca(OH)_2$ 90/20	9.6 ± 2.5	956 ± 225	15.1 ± 2.9	7
[b](SD 25/75)/$Ca(OH)_2$ 90/30	2.2 ± 0.7	181 ± 51	10.2 ± 4.6	8

The BA and C_{max} of set[a] was compared to set[b] using Contrast Analysis (***$P \leqslant 0.001$).

Table 3
Elasticity (G') and viscosity (G'') of 10% (w/w) dispersions in simulated nasal fluid

Formulation	G' (Pa)	G'' (Pa)
SD 25/75	1699 ± 31	142 ± 4
(SD 25/75)/$Ca(OH)_2$ 90/1	1673 ± 49	136 ± 3
(SD 25/75)/$Ca(OH)_2$ 90/10	1822 ± 26	152 ± 6
(SD 25/75)/$Ca(OH)_2$ 90/20	1412 ± 176	113 ± 15
(SD 25/75)/$Ca(OH)_2$ 90/30	–	–

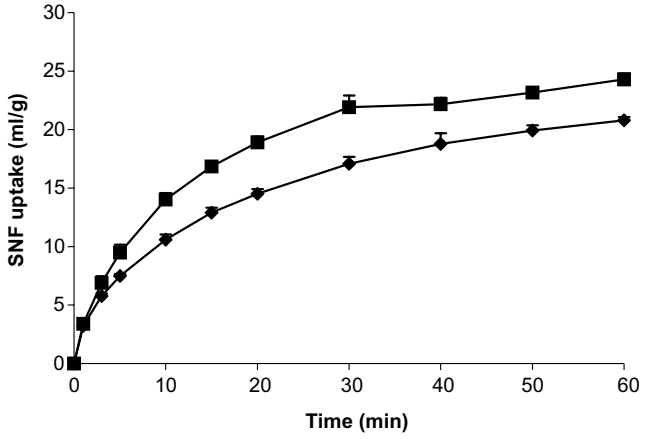

Fig. 3. Uptake of simulated nasal fluid by SD 25/75 (♦) and (SD 25/75)/$Ca(OH)_2$ 90/10 (■) powders ($n = 3$, mean ± SD).

network, hence the viscosity and elasticity after dispersion in the nasal fluid could not be measured. This would result in a faster clearance of the formulations from the nasal cavity, resulting in a shorter t_{max}.

The formulation based on (SD 25/75)/$Ca(OH)_2$ 90/10 had a higher elasticity after dispersion in the nasal fluid compared to the reference formulation without $Ca(OH)_2$ (Table 3). Although this can provide an explanation for the enhanced absorption observed for the formulation, it does not explain the shorter t_{max}. An enhanced viscosity and elasticity of a formulation is generally associated with a reduced mucociliary clearance whereby the residence time of the formulation in the nasal cavity is extended what may result in a prolonged drug absorption [6]. Furthermore, this formulation had a higher and faster absorption capacity of simulated nasal fluid what might result in a higher paracellular transport (Fig. 3), another factor contributing to the higher and faster absorption of insulin in comparison to the formulation without $Ca(OH)_2$. The maximum in insulin absorption observed for (SD 25/75)/$Ca(OH)_2$ 90/10 is probably due to an optimal balance between Na^+- and Ca^{2+}-carboxylate groups on the poly(acrylate) chains.

The strong interaction between Ca^{2+} and the carboxylate groups was confirmed during in vivo analysis. Due to the low concentration of free Ca^{2+}-ions, the extracellular Ca^{2+}-concentration in the nasal cavity does not increase. A higher extracellular concentration of divalent cations would modulate the integrity of the intercellular junctions and reduce the paracellular absorption of insulin through the epithelium via the tight junctions [22,23]. In contrast, a higher C_{max} was observed in the formulations based on the ratio 90/1, 90/10 and 90/20 in comparison with the reference formulation without $Ca(OH)_2$, indicating that the concentration of free Ca^{2+}-ions was low. On the other hand, a progressive dissociation of Ca^{2+} from Ca^{2+}-carboxylate in these formulations might be responsible for their shorter t_{max} due to the closing of the tight junctions in response to the increase of Ca^{2+}-concentration in the nasal fluid [22,23].

4. Conclusion

The present study demonstrated that the nasal absorption of insulin could be improved via the incorporation of $Ca(OH)_2$ in a powder formulation based on starch and poly(acrylic acid) (neutralised with NaOH). The formation of a mixture of sodium and calcium carboxylate salts of poly(acrylic acid) was a key factor determining the nasal bioavailability of insulin.

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