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ORIGINAL ARTICLE

In vitro evaluation of gentamicin- and vancomycin-containing minitablets as a replacement for fortified eye drops

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Abstract

Objective: Ocular bioadhesive minitablets containing gentamicin and vancomycin were developed using different powder mixtures of pregelatinized starch and Carbopol (physical or cospray-dried mixtures). *Methods*: Drug content, antimicrobial activity, and radical formation of the powders used for tablet preparation were evaluated immediately and 30 days after gamma sterilization. Tablet properties and in vitro drug release from the sterilized minitablets were determined. Storage stability of vancomycin and gentamicin in sterilized bioadhesive mixtures was examined by LC–UV/MS and a microbiological assay, respectively. A bioadhesive powder mixture containing only vancomycin was irradiated by X electromagnetic radiation to evaluate vancomycin stability following sterilization through irradiation. *Results*: The antimicrobial activity of gentamicin against *Staphylococcus epidermidis* was not altered in comparison to nonsterilized formulations. Only after an overkill dose of 50 kGy, the concentration of vancomycin decreases to an extent that was pharmaceutically significant. No significant difference in radiation stability between drug substance and product (i.e., powder mixture) was observed. A shift in stability profile was not observed at 6 weeks after irradiation. All other degradation products were present only in small quantities not exceeding 1.0%. The in vitro drug release from the minitablets prepared with physical powder mixtures of pregelatinized starch and Carbopol® 974P NF (96 : 4) was faster compared to the cospray-dried mixtures of starch with Carbopol® 974P NF (ratio: 95:5 and 85:15). The electron paramagnetic resonance signals of the radicals formed during sterilization were still visible after storage for 30 days. The slug mucosal irritation test indicated mild irritation properties of the bioadhesive powder mixtures although no tissue damage was observed.

Key words: *EPR; gentamicin; irritation study; ocular minitablet; radiostability; vancomycin*

Introduction

Bacterial keratitis is a serious ophthalmological disease that can result in permanent loss of vision if an appropriate therapy is not instituted in a timely manner[1,2]. Standard treatment of severe bacterial keratitis consists initially of frequent instillation of eye drops containing a broad-spectrum antimicrobial agent. The drop application schedule requires strict discipline from the patient or care provider. Selection of a potent agent or agents with a broad-activity spectrum against the most common corneal pathogens is preferred[3]. At present the following two regimens are commonly used: combination therapy with fortified antibiotics (e.g., an aminoglycoside with vancomycin hydrochloride) and monotherapy with a fluoroquinolone (e.g., ciprofloxacin)[4–6]. Vancomycin is

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a glycopeptide antibiotic active against a large number of Gram-positive cocci and bacilli[7]. The aminoglycoside antibiotic gentamicin is widely used to treat bacterial infections owing to its adequate spectrum against Gram-negative bacteria[8,9]. Furthermore, vancomycin and aminoglycoside antibiotics have shown synergism against many organisms[10–13] and are therefore selected as model drugs.

The bioavailability of drugs applied through eye drops is poor because of rapid precorneal clearance, induced lacrimation, and normal tear turnover[14]. This leads to frequent instillation of a highly concentrated solution to achieve the desired therapeutic effect. A high instillation frequency is, however, associated with poor patient compliance, and the systemic absorption of drug drained through the nasolachrymal duct may result in some undesirable side effects[15–18]. Moreover, the frequent use of concentrated solutions containing preservatives may lead to toxic side effects and cellular damage at the ocular surface[19,20]. Therefore, numerous strategies have been developed to increase the bioavailability of the drug applied by prolonging the contact time between the drug and the corneal/conjunctival epithelium. In an attempt to optimize the dosage form, films and inserts allowing a slow drug release over a long period of time were developed[21]. These single-use ophthalmic dosage forms are effective and require less frequent administration[22].

Ocular bioerodible minitablets containing sodium fluorescein (as diagnostic agent) or ciprofloxacin (as antimicrobial agent) in combination with a mixture of starch and polyacrylic acid (Carbopol) (used as bioadhesive excipients) have been developed to obtain sustained drug release. In vivo studies demonstrated that the formulations were well accepted by healthy volunteers[5,23–25]. Furthermore, Choonara et al.[26,27] developed doughnut-shaped minitablet formulations by using poly(lactide-co-glycolide) polymer for intraocular delivery of foscarnet and ganciclovir to obtain prolonged drug delivery.

In this study, ocular minitablets containing gentamicin and vancomycin were prepared using a physical or cospray-dried mixture of bioadhesive polymers (starch and polyacrylic acid) to achieve prolonged drug release in the tear film after application of the minitablet in the cul-de-sac. As only sterile minitablets can be applied to the eye drug content, radical formation [using electron paramagnetic resonance (EPR)] and irritation potential [using the slug mucosal irritation (SMI) test] of the powder mixtures were determined after sterilization by gamma (γ)-irradiation. In addition, the short-term storage stability of vancomycin and gentamicin in gamma-irradiated bioadhesive mixtures was determined by LC–UV/MS and a microbiological assay, respectively. Crushing strength, friability, water uptake, and in vitro drug release were evaluated as in vitro characteristics of the minitablets.

Materials and methods

Materials

Polyacrylic acid (Carbopol® 974P NF) and drum-dried waxy maize (DDWM) starch were supplied by Noveon (Cleveland, OH, USA) and Cerestar (Vilvoorde, Belgium), respectively. The cospray-dried mixtures of Amioca® starch and Carbopol® 974P NF (95 : 5 and 85 : 15, w/w) were received from National Starch and Chemical Company (Bridgewater, NJ, USA). Both DDWM and Amioca® are pregelatinized high-amylopectin starches.

Sodium 1-heptanesulfonate, thioglycolic acid, triethylamine, and *o*-phthalaldehyde were obtained from Sigma-Aldrich Chemie (Steinheim, Germany). Gentamicin sulfate and vancomycin hydrochloride were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Sodium stearyl fumarate (as a lubricant) was a gift of Edward Mendell Co. (New York, NY, USA). Tetrahydrofuran, acetonitrile, sodium dihydrogen phosphate dihydrate ($NaH_2PO_4.2H_2O$), and disodium hydrogen phosphate dihydrate ($Na_2HPO_4.2H_2O$) were purchased from Merck (Darmstadt, Germany) and were used to prepare phosphate-buffered saline (PBS, pH 7.4). Salts for the preparation of simulated lachrymal fluid (SLF) were obtained from Merck (Leuven, Belgium) (KCl and NaCl), Merck (Darmstadt, Germany) ($NaHCO_3$), and Sigma Chemicals Co. (St. Louis, MO, USA) ($CaCl_2$ and $MgCl_2$). SLF is an electrolyte solution composed of 1.7893 g/L KCl, 6.3118 g/L NaCl, 2.1842 g/L $NaHCO_3$, 0.0670 g/L $CaCl_2.2H_2O$, 0.1572 g/L $MgCl_2.6H_2O$, adjusted with 0.1 N HCl to a pH of 7.4 ± 0.1[28]. Purified water produced by Milli-Q (Millipore, Bedford, MA, USA) was used throughout the experiments. All organic solvents were of analytical grade and were used without further purification.

Methods

Production of minitablets

The compositions of the minitablets used in this study are presented in Table 1. The drug content (2% and 5%), the starch/Carbopol ratio (96:4, 95:5, and 85:15, w/w), and the manufacturing procedure of the bioadhesive mixture (physical mixture versus cospray-dried mixture) were included as formulation variables. The powders (without drugs) were first mixed with mortar and pestle and afterward blended in a tumbling mixer for 10 minutes (Turbula T2A, Willy A. Bachoffen Maschinenfabrik, Basel, Switzerland). Because of the poor flowing properties and low bulk density of these

Table 1. Composition of the formulations evaluated in this study.

Code	PM96/4-2%	PM96/4-5%	CS85/15-2%	CS85/15-5%	CS95/5-5%	CS95/5-5%-V[a]	CS95/5-5%-G[a]
DDWM	91.20	85.44	–	–	–	–	–
Amioca	–	–	80.65	75.55	84.55	89.30	89.30
Carbopol	3.80	3.56	14.35	13.45	4.45	4.70	4.70
Gentamicin sulfate	2	5	2	5	5	–	5
Vancomycin HCl	2	5	2	5	5	5	–
Sodium stearyl fumarate	1	1	1	1	1	1	1

PM, physical mixtures of DDWM and Carbopol; CS, cospray-dried mixture of Amioca and Carbopol.
[a]Only powder mixtures were prepared for this formulation, no minitablets.

starch/Carbopol mixtures, it was necessary to prepare granules by slugging to obtain high-quality minitablets. Large tablets (~13 mm, 250 mg) were compressed at 5 kN using an eccentric tabletting machine (Korsch EKO, Berlin, Germany). The tablets were crushed in a mortar and the granules obtained were sieved on a Retsch VE 1000 shaker (Retsch, Haan, Germany) to isolate the fraction $F_{90–250\ \mu m}$. This granule fraction was blended with gentamicin sulfate and vancomycin hydrochloride in a tumbling mixer (mixing time: 10 minutes). The resulting powder mixture was compressed into minitablets (7 mg) at a compression force of 0.5 kN, using an eccentric compression machine (Korsch EKO) equipped with four concave punches (diameter 2 mm).

The minitablets were sterilized by gamma irradiation using a ^{60}Co source (Gammir-I-Sulzer irradiator unicell, IBA-Mediris/Sterigenics, Fleurus, Belgium). The dose rate was set at 1.0 kGy/h and the irradiation dose applied was 25 kGy. All experiments were carried out at room temperature.

Characterization of minitablets

The following in vitro parameters of the minitablets were evaluated: crushing strength, friability, water uptake, and drug release.

Crushing strength. The crushing strength of the minitablets (n = 10) was analyzed using an instrumented uniaxial press (L1000R; Lloyd Instruments, Segensworth, Fareham, UK), equipped with a 20 N load cell.

Friability. The friability of the minitablets was determined by subjecting 10 tablets together with 100 glass beads (average diameter of 4 mm) to falling shocks for 10 minutes in an Erweka friabilator (TA3, Offenbach/Main, Germany), set at a speed of 25 rpm. After 10 minutes the glass beads were removed. The weight loss of the tablets was determined to calculate the friability[24].

Water uptake. The water uptake at room temperature was studied gravimetrically. The minitablets (mass = m_d) were placed on the upper side of a filter that was connected on its lower side to a reservoir filled with SLF. The weight of the swollen minitablet (m_w) was determined at 120 and 480 minutes to calculate the water uptake of the minitablets (W) as follows:

$$W = \frac{m_w - m_d}{m_d}. \quad (1)$$

In vitro drug release. The release of gentamicin sulfate and vancomycin hydrochloride from the minitablets (n = 4) was evaluated using glass vials in an oscillating water bath[24,25]. A minitablet was accurately weighed and transferred to a glass vial containing 1 mL SLF. To avoid water evaporation, the vial was covered with a rubber cap and placed in an oscillating water bath at 32 ± 1°C. In the course of the experiment, aliquots of 80 µL were withdrawn after 0, 60, 120, 180, 240, 300, 360, and 1440 minutes and replaced by an equal volume of SLF. The samples were diluted to 1 mL with SLF and centrifuged at 1420 × gh for 10 minutes. The concentrations of gentamicin sulfate and vancomycin hydrochloride in the supernatant were determined by high-performance liquid chromatography (HPLC). The percentage released at each time point was expressed as a fraction of the total amount released after 24 hours.

The HPLC system consisted of a Gilson 321 pump (Gilson, Villiers-le-Bel, France), a UV-VIS 152 detector (Gilson), a µBondapack™ C_{18} column (5 mm × 10 cm, 5 µm particle size) (Waters, Milford, MA, USA) for gentamicin sulfate, a µBondapack™ C_{18} column (4.6 mm × 25 cm, 5 µm particle size) (Waters) for vancomycin hydrochloride, and an HP 3395 integrator (Hewlett–Packard Company, Palo Alto, CA, USA). The mobile phase and other conditions used correspond to the monograph of the European Pharmacopoeia[29]. Gentamicin sulfate and vancomycin hydrochloride were detected at 330 and 280 nm, respectively. The linearity, sensitivity, precision, accuracy, and specificity were determined. The limit of detection and the limit of quantification for gentamicin sulfate and vancomycin hydrochloride were 5 and 7.5 µg/mL and 10 and 12.5 µg/mL, respectively.

Slug mucosal irritation test

To evaluate the eye irritation of the formulations, the SMI test was used[5,30]. The principle of the SMI test is based on the fact that slugs exposed to irritating substances produce mucus to protect the body wall[31]. The original procedure was modified for the evaluation of the irritation potential of bioadhesive powder mixtures[32,33]. Slugs [Arion lusitanicus (Mabille)] weighing 3–6 g were isolated 2 days before the start of an experiment and were placed in a vented plastic box lined with a paper towel, moistened with PBS (pH 7.4) at 18–22°C. Slugs treated with DDWM were used as negative controls, whereas slugs treated with DDWM/sodium lauryl sulfate (SLS) (80:20) were positive controls. The slugs were placed daily on 20 mg powder for 30 minutes during five consecutive days. For each powder formulation five slugs were used. The reduction in body weight caused by the treatment was measured by weighing the slugs before and after the 30-minute contact period and expressed as a percentage (w/w) of the initial body weight. The amount of mucus produced during each contact period was measured by weighing the Petri dishes with the test substance before and after the 30-minute contact period. The mucus production (MP) was expressed as percentage of the body weight (w/w).

After each 30-minute contact period, the slugs were transferred to a fresh Petri dish and 1 mL PBS was added. After 1 hour, the PBS was collected with a micropipette and the slugs were placed in a fresh Petri dish and again 1 mL PBS was added. One hour later, the PBS was collected. After sampling, the slugs were placed on a membrane filter (cellulose acetate 0.45 µm; Sartorius, Goettingen, Germany) moistened with 2 mL PBS in a Petri dish until the next contact period. The PBS samples were analyzed for the presence of proteins, lactate dehydrogenase (LDH, EC 1.1.1.27), and alkaline phosphatase (ALP, EC 3.1.3.1) released from the body wall. The protein concentration in the PBS samples was determined with a NanoOrange™ protein quantitation kit (Molecular Probes, Leiden, the Netherlands) and expressed in µg/mL/g body weight. The LDH and ALP activity was measured with an enzyme kit (LDH/HBDH 2.8 and ALP, respectively; ABX Diagnostica, Montpellier, France) and expressed in IU/L/g body weight.

The total MP, the mean protein release (without data of the first day of treatment), and the mean LDH release of each slug as well as the number of slugs showing ALP release were calculated. Based on these endpoints, the irritation potential and the tissue damage induced by a formulation were estimated using a classification prediction model[34]. A formulation is considered toxic in case of mortality. For the assessment of the irritation potential, cut-off values for the total MP are used to classify solid compounds into nonirritant (total MP <7%), mildly (total MP 7–12%), moderately (total MP 12–20%), and severely (total MP > 20%) irritant. Furthermore, for the prediction of tissue damage, a decision tree combining the results of the mean protein, mean LDH, and the number of slugs showing ALP release is used to convert the results into tissue damage grades: no, slight, moderate, and severe tissue damage[34].

Evaluation of the radicals after gamma irradiation

The amount of radicals induced by gamma sterilization (25 kGy) of gentamicin sulfate, vancomycin HCl, excipients, and powder mixtures and the kinetics of radical disappearance during storage of these products were investigated by EPR. The EPR spectrometer used was the Bruker EMS104 portable spectrometer. The EPR spectrometer operates in X-band, more specifically with a microwave frequency of 9.77 GHz, and can record EPR resonances around $g = 2$. The maximum magnetic field range is 20.0 mT, which covers to a sufficient extent all of the EPR signal ranges encountered in EPR dosimetry. For the actual EPR measurements, the following parameters were set on the spectrometer: microwave power 4.99 mW, modulation frequency 100 kHz, modulation amplitude 0.1 mT, center magnetic field (B_0) 349.0 mT, scan range 16.5 mT, sweep time 83.9 seconds, and time constant 81.92 ms. The EPR spectra of the powders were recorded at room temperature. EPR spectra are usually recorded in derivative mode, which means that the intensity of an EPR signal is obtained by calculating the double integral of the spectrum. In practice, however, the peak-to-peak signal height in the EPR spectrum is taken as a measure of the number of radicals. All EPR experiments were carried out in triplicate.

Vancomycin stability after irradiation

The bulk drug substance vancomycin as well as a bioadhesive powder mixture (drug product) containing 5% (w/w) vancomycin HCl (formulation CS95/5-5%-V) was subjected to radiation receiving a European Pharmacopoeia-recommended sterilization dose of 25 kGy and an overkill dose of 50 kGy. Irradiations were performed at the Department of Subatomic and Radiation Physics of Ghent University, with following conditions: an electron beam with a mean electron energy of 10 MeV was focused on a Tantalum target, resulting in a mean X electromagnetic radiation energy of 1.5 MeV, similar to a conventional ^{60}Co sterilization source. Samples were irradiated for 2 hours (25 kGy) and 4 hours (50 kGy).

The nonirradiated drug substance and drug product were also subjected to heat treatment, that is, 2 and 4 hours at 50°C, thereby simulating the heat experienced by the samples at 25 and 50 kGy irradiations, respectively.

Quantitative HPLC for vancomycin assay was performed with a Waters Breeze HPLC system, equipped with a 25 µL loop; detection was performed with a Waters 2487 dual λ absorbance detector and Breeze software (Waters Corporation). The HPLC method used was an isocratic system based on the related substances test for vancomycin B in the European Pharmacopoeia monograph[29]. The mobile phase consisted of tetrahydrofuran/acetonitrile/water containing 0.4% triethylamine (brought to pH 3.2 with phosphoric acid) (1:7:92, v/v/v) and the flow was set at 1.2 mL/min. Detection was by UV absorbance at 280 nm. A Lichrospher 100 RP 18 (5 µm; 4.0 × 250 mm) column was used. The column was kept at room temperature (21 ± 3°C). Results are expressed as percentage found relative to the theoretical quantity. The overall SD of the results was calculated from repeat analyses ($n = 6$) and found to be 2.5%.

LC–UV/MS was performed with an apparatus consisting of a Spectra System SN4000 interface, a Spectra System SCM1000 degasser, a Spectra System P1000XR pump, a Spectra System AS3000 auto sampler, and a Finnigan LCQ classic ion trap equipped with an electron spray ionization source in positive mode (Thermo, San Jose, CA, USA) equipped with a SPD-10A UV-VIS detector set at 285 nm (Shimadzu, Kyoto, Japan) and Xcalibur 1.2 software (Thermo) for data acquisition. The mobile phase used in the LC–MS experiments, according to Diana et al.[35,36], consisted of methanol:water:0.2 M NH_4OAc (the ammonium acetate solution was brought to pH 9.0 by adding concentrated ammonia) (30:65:5, v/v/v). A Zorbax extended C18 (250 × 3.0 mm i.d., 5 µm; Agilent Technologies, Waldbronn, Germany) kept at room temperature was used with a flow of 150 µL/min.

In addition, for qualitative control purposes, forced degradation solutions of vancomycin were prepared of 0.05 mg/mL vancomycin hydrochloride in 0.05 M HCl and 15% H_2O_2. These solutions were kept at 60°C for 4 hours before being injected into the LC–MS system.

Storage stability of vancomycin and gentamicin in gamma-irradiated bioadhesive mixtures

The short-term storage stability (21 ± 3°C) of gentamicin sulfate and vancomycin hydrochloride in a bioadhesive powder (formulation CS95/5-5%-G or CS95/5-5%-V) was evaluated following irradiation. After applying two different doses (i.e., 10 and 25 kGy) using a ^{60}Co source (Gammir-I-Sulzer irradiator unicell; IBA-Mediris/Sterigenics, Fleurus, Belgium), quantitative LC–UV assay determinations for vancomycin as described above were performed within 1 and 4 weeks after irradiation. The concentration of gentamicin sulfate in the bioadhesive powder mixtures was determined using a microbiological assay immediately after irradiation and after 4 weeks of storage (21 ± 3°C). An agar diffusion method with *Staphylococcus epidermidis* ATCC 12228 as test bacterium was used, according to the European Pharmacopoeia[29]. The same method was used for the determination of the concentration of gentamicin sulfate in the minitablets. Commercial gentamicin sulfate (Sigma Aldrich) was used as a standard. A stock solution of gentamicin sulfate was prepared in 0.1 M sodium phosphate buffer (pH 8.0) and further diluted with the same buffer to yield working solutions containing 3.0 and 0.75 µg gentamicin sulfate per mL, respectively.

The nonactive ingredients of the powder mixtures were also added to the standard solutions to compensate for their possible effect on the diffusion of gentamicin sulfate.

A concentrated bacterial suspension was divided into 1-mL vials and stored in liquid nitrogen. After thawing 9 mL of peptone, water was added for reconstitution. Of this suspension, 2.5 mL was mixed with 250 mL Antibiotic medium no. 11 (pH 8.0) (BD) and poured on a glass plate in an aluminum frame (30.5 × 30.5 cm).

Thirty-six holes (9 mm diameter) were punched out from the agar. Each hole was filled with 200 µL of the gentamicin sulfate working or standard solutions. The plates were subsequently incubated at 37°C for 18 hours and the obtained inhibition zones were measured by means of the Microbiological Assay System (Scientific and Technical Supplies, Newmarket, Suffolk, UK) consisting of a projector and a measuring unit. Calculations were made by a computer program of the Assay System.

Antibacterial activity

To determine whether gamma-irradiated sterilization had an influence on the antibacterial activity of gentamicin sulfate and vancomycin hydrochloride in the minitablets, microbiological assays were used with *Staphylococcus epidermidis* ATTC 12228 and *Bacillus subtilis* ATTC 6633 as test bacteria[29]. To release the drugs from the gamma-irradiated minitablets, sterile water was added to three minitablets and the samples were placed in an ultrasonic bath (Julabo USR3; Julabo, Seelbach, Germany) for 3 hours. Stock solutions were subsequently diluted in a phosphate buffer (0.1 M, pH 8) to obtain gentamicin sulfate or vancomycin hydrochloride working concentrations of 2 and 10 µg/mL, respectively. Nonsterilized minitablets were also included and were used to prepare the reference solutions. They were treated identically as the sterilized minitablets and equal concentrations of gentamicin and vancomycin were prepared.

Antibiotic Medium 11 (Difco Laboratories) was seeded with a suspension of each test strain, containing approximately 10^6 CFU/mL. The rest of the procedure was carried out as described above. The activity of the irradiated tablets was expressed as a percentage of the activity detected in nonirradiated samples.

Statistical analysis

Statistical analysis of data was performed with a *t*-test or one-way analysis of variance using Statistica® software (Statsoft, Tulsa, OK, USA).

Results and discussion

Evaluation of the minitablets

The physical properties of gamma-sterilized minitablets prepared using physical as well as cospray-dried powder mixtures are summarized in Table 2. The crushing strength of the minitablets was higher when cospray-dried powder mixtures were used ($P < 0.05$) as cospray-drying of powders can change the physical properties of starch, resulting in a higher deformability and stronger interparticle bonding[37]. Furthermore, lowering the amount of Carbopol® 974 P in the cospray-dried powder mixture increased the crushing strength of the minitablets ($P < 0.05$). The friability of all minitablets prepared was below 1% (w/w).

The results of water sorption by the samples are shown in Figure 1. The water uptake of all formulations was higher ($P < 0.05$) when increasing the contact time of minitablets with PBS from 2 to 8 hours. Furthermore, the water uptake rate during the first 2 hours was higher than the value obtained between 2 and 8 hours ($P < 0.05$), indicating a slower water uptake rate for all the formulations during the latter stages of the test. No significant changes ($P < 0.05$) were observed in water uptake of the minitablets at 2 and 8 hours. Gamma irradiation had no statistically significant influence on the water uptake behavior of all minitablets, except for formulation PM96/4-5% ($P > 0.05$). In contrast, Weyenberg et al.[38] reported a decreased and slower water uptake by minitablets prepared with pregelatinized starch and Carbopol® 974P after sterilization by gamma sterilization because of a decrease of the amylopectin fraction, which is responsible for the swelling and rheological properties of starch.

The antimicrobial activities of gentamicin sulfate or vancomycin hydrochloride in sterilized minitablets against *S. epidermidis* and *B. subtilis* were not altered in comparison to the nonsterilized samples ($P > 0.05$) (Table 2).

The in vitro release of gentamicin sulfate and vancomycin hydrochloride from different minitablets is presented in Figures 2 and 3, respectively. For both drugs, the drug release rate during the initial phase of dissolution testing was faster compared to the latter phase. This correlated with the initial higher water uptake rate (Figure 1). Furthermore, upon wetting the polymers changed from a

Table 2. Characteristics and in vitro antimicrobial activity of the minitablets containing gentamicin sulfate and vancomycin hydrochloride (mean ± SD).

Formulation	Crushing strength (*N*) ($n = 10$)	Friability (%) ($n = 10$)	Antimicrobial activity (%)[a] against	
			Staphylococcus epidermidis	*Bacillus subtilis*
PM96/4-2%	11.6 ± 1.3	0.65 ± 0.08	100.4 ± 1.6	98.8 ± 0.4
PM96/4-5%	13.8 ± 3.1	0.41 ± 0.13	99.7 ± 1.4	99.2 ± 2.3
CS85/15-2%	17.4 ± 2.5	0.09 ± 0.16	98.5 ± 2.1	100.3 ± 1.1
CS85/15-5%	20.2 ± 3.4	0.30 ± 0.15	100.1 ± 0.6	99.9 ± 0.8
CS95/5-5%	24.6 ± 2.4	0.34 ± 0.22	101.3 ± 1.1	100.2 ± 0.5

[a]Expressed as percentage of the activity of nonsterilized minitablets (mean ± SD, $n = 3$).

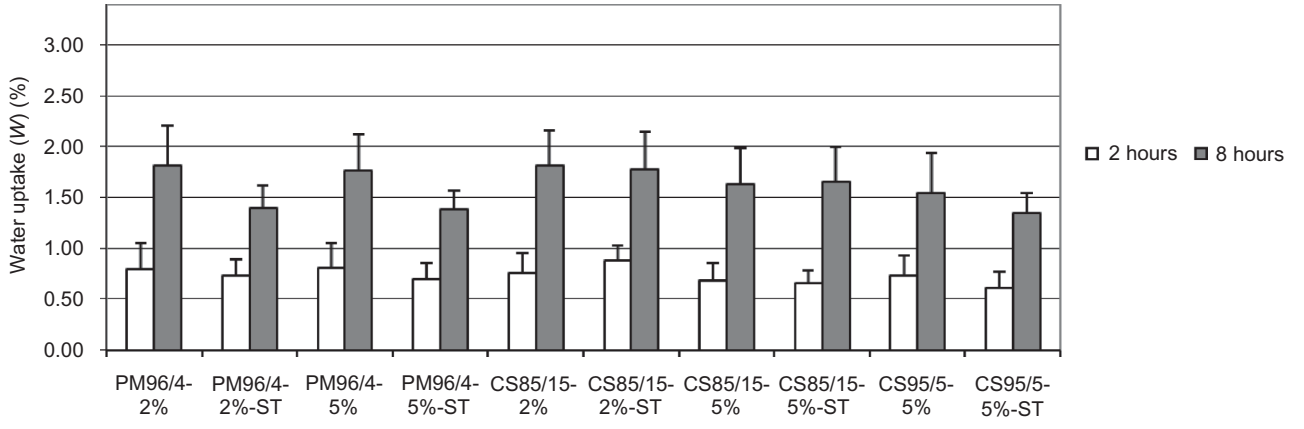

Figure 1. Water uptake of minitablets prepared using different bioadhesive powder mixtures (mean ± SD, $n = 3$). ST, sterilized minitablets.

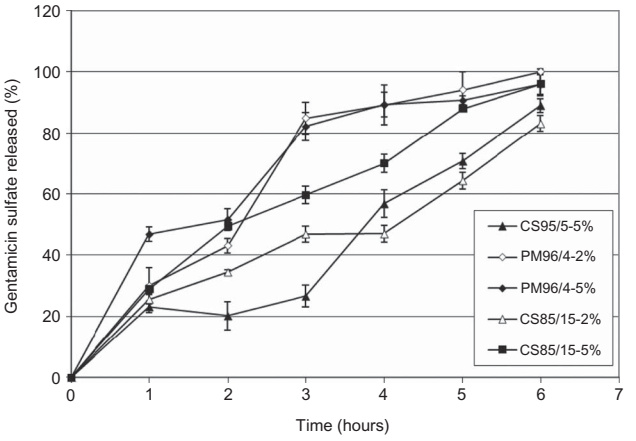

Figure 2. In vitro release of gentamicin sulfate from minitablets prepared using different bioadhesive powder mixtures (mean ± SD, $n = 4$).

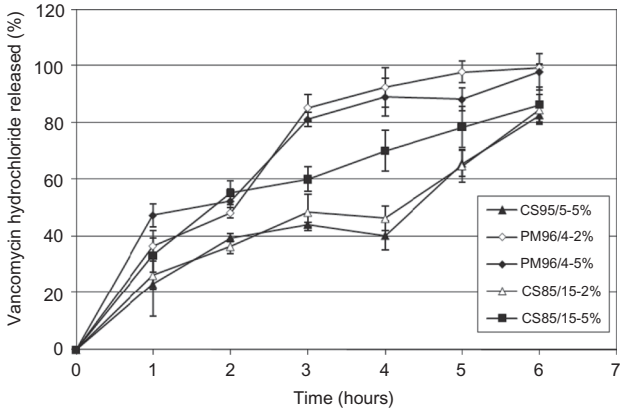

Figure 3. In vitro release of vancomycin hydrochloride from the minitablets prepared using different bioadhesive powder mixtures (mean ± SD, $n = 4$).

glassy to a rubbery state and a gel structure is rapidly formed around the tablet matrix, thus decreasing drug diffusion through this gel barrier into the bulk phase[39].

The average release rate of gentamicin sulfate from the minitablets prepared by cospray-dried Amioca® starch with Carbopol® 974P was slower compared to the physical mixtures of DDWM with Carbopol® 974P because of the different rheological behavior of these mixtures. Bozdag et al.[40] reported that the elastic properties of dispersions prepared with cospray-dried Amioca® starch with Carbopol® 974P powder mixtures were higher in comparison to physical blends of DDWM and Carbopol® 974P. An increase of the viscoelastic properties of the dispersion would result in a lower diffusion rate of the drug from the gelling tablet. Furthermore, when the amount of Carbopol® 974P was increased in the cospray-dried mixture (CS85), a slower release rate was observed because of the higher elasticity of these formulations[40]. Phinney et al.[41] prepared gentamicin- and vancomycin-loaded collagen shields for the treatment of bacterial keratitis and evaluated the in vitro and in vivo drug release behavior from these shields. For in vitro release studies, the authors reported that presoaked collagen shields released a large part of gentamicin within the first 30 minutes, whereas vancomycin was released gradually over a period of 6 hours. In our study, however, both of the drugs were released slowly during 6 hours which is an advantage for providing sustained drug release in the eye to achieve increased drug bioavailability.

Vancomycin stability after gamma irradiation

Assay results of vancomycin after the different treatments (two heat treatments and two radiation treatments) are presented in Table 3.

A decreasing trend in HPLC–UV assay values was observed for drug substance as well as drug product (bioadhesive powder mixture) after radio-sterilization. Nevertheless, even the samples irradiated at a 50 kGy 'overkill' dose were still close to the 93% lower limit of the quality specifications required by the European Pharmacopeia monograph for the drug substance. No significant difference between drug substance and drug product is observed.

Chromatograms obtained with the LC–UV/MS method, of untreated vancomycin hydrochloride, 50 kGy irradiated vancomycin hydrochloride, and the stress degradations are given in Figure 4, with the inset showing the recorded mass spectra at retention time 15.05 minutes (peak v with $[M + H]^+$ $m/z = 1448$, corresponding to vancomycin) and its major degradation product observed at 19.35 minutes (peak b with $[M + H]^+$ $m/z = 1305$, corresponding to desvancosaminyl vancomycin B, at a concentration of 3.4% in the 50 kGy irradiated drug substance, but also present in untreated sample at 1.2%). Several minor radiation-originating degradation products were detected by LC–UV/MS. In Figure 4, the most important ones are denoted peak (a) (m/z of $[M + H]^+ = 1412$, at a concentration of 0.6%), peak c (m/z of $[M + H]^+ = 1463$, at a concentration of

Table 3. LC–UV assay results of vancomycin hydrochloride.

	Assay results[a] (%)	
Treatment	Drug substance	Drug product
25 kGy	94.5	97.3
50 kGy	91.0	92.1
2 hours at 50°C	96.5	97.8
4 hours at 50°C	98.1	99.2

Drug substance, pure vancomycin hydrochloride; drug product, bioadhesive powder mixture containing 5% (w/w) vancomycin HCl (formulation CS95/5-5%-V).

[a]Assay results after treatment are expressed in relation the results obtained after analysis of the same sample without treatment.

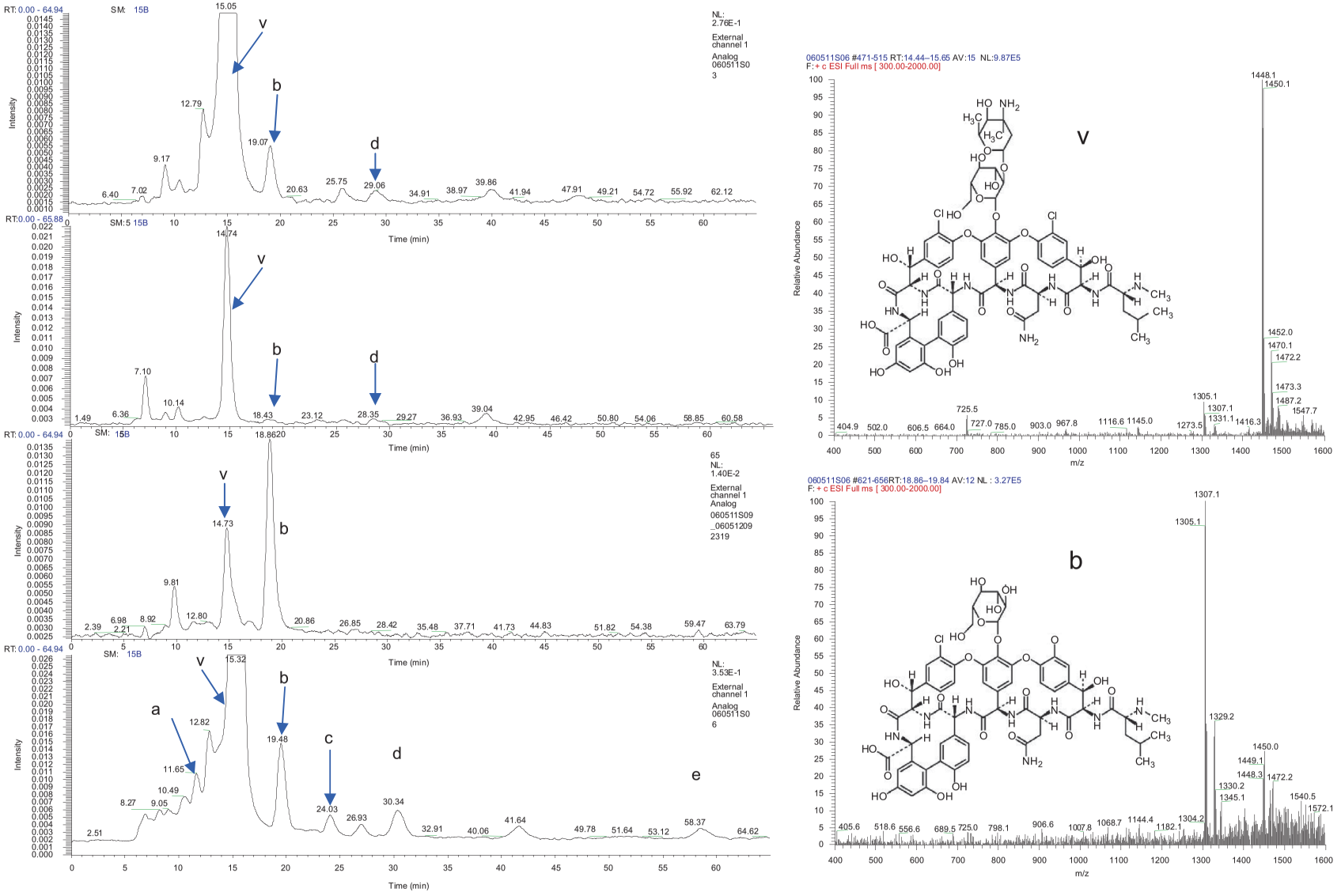

Figure 4. From top to bottom: UV chromatograms of untreated vancomycin (±1.25 mg/mL), H_2O_2 treated (0.05 mg/mL), HCl treated (0.05 mg/mL), and 50 kGy irradiated vancomycin (±2 mg/mL). Mass spectra of vancomycin peak (v) and desvancosaminylvancomycin peak (b). Peaks a, c, and d identify minor degradation products originating from the radiation of vancomycin.

0.8%), peak d (m/z of $[M + H]^+$ = 1428 and 1463, at a concentration of 1.7%, m/z of $[M + H]^+$ = 1428 was also present at 0.7% in untreated sample), and peak e (m/z = 1428 of $[M + H]^+$ at a concentration of 1%). As the low concentration required no further identification and based on the objective of this study, no impurity reference injections nor detailed structural clarification was performed. Other groups are currently investigating possible related impurities, however, originating from the synthesis[35,36]. The related impurity peaks after the 50 kGy overkill irradiation treatment that exceeded 1.0% were already present in the untreated samples, compliant with the European Pharmacopoeia quality limit of maximal 4.0% individual impurity. Therefore, no qualification issue was expected with these compounds. The other degradation peaks observed were lower or equal to 1%, which is the qualification threshold for an envisaged maximal daily dose of 10 mg chemically synthesized compound, which is considered stricter than for a nature-derived antibiotic as vancomycin (CPMP/ICH/2738/99-R2)[42]. Our findings are thus consistent with recent observations that the major radiation-degradation compounds of drugs were not unique to irradiation[42]. Moreover, these findings confirmed those of Bartolotta et al.[43], who used a nonspecific UV assay method.

EPR study

The EPR signals of the pure powders and the powder mixtures containing gentamicin sulfate and vancomycin hydrochloride at room temperature after irradiation (25 kGy) were monitored by determining the peak-to-peak height of the spectrum as a function of time (days 1 and 30) (Figure 5). No EPR signal was found in the non-irradiated samples. The highest signals were recorded in the gentamicin sulfate sample at days 1 and 30. No data were available in the literature concerning EPR studies of gentamicin sulfate and vancomycin hydrochloride.

A decrease in the amount of radicals was observed in all samples at 30 days and this was significant ($P < 0.05$) for gentamicin sulfate, vancomycin hydrochloride, and the physical powder mixtures. The peak-to-peak heights were 22.3, 9.8, 7.4, and 6.5 for gentamicin sulfate, vancomycin hydrochloride, PM96/4-2%, and PM96/4-5%, respectively, at day 1, whereas they had dropped to 5.6, 5.4, 4.0, and 3.8, respectively, after day 30.

The EPR signals of the radicals formed were still visible even after storage for 30 days. Furthermore, the experimental peak-to-peak heights were the lowest for the excipients used in the minitablets. These results are in agreement with the work of Weyenberg et al.[25], who evaluated the effect of different sterilization methods on the properties of bioadhesive powders and minitablets.

Although the EPR signal in the bioadhesive formulations was still visible after 30 days of storage, the radicals in the powder did not affect the drug content, because no significant assay shifts were observed 4 weeks after irradiation (Table 4). However, the impurity profile of vancomycin showed a relative increase in major degradation product desvancosaminyl vancomycin of 0.4% for 10 kGy treatment and 0.7% for 25 kGy after 6 weeks of storage at room temperature.

SMI test

The effects of the repeated treatment with the powders on the endpoints of the SMI test are presented in Table 5. The total MP of the slugs treated with vancomycin hydrochloride was the highest of all powders tested, indicating

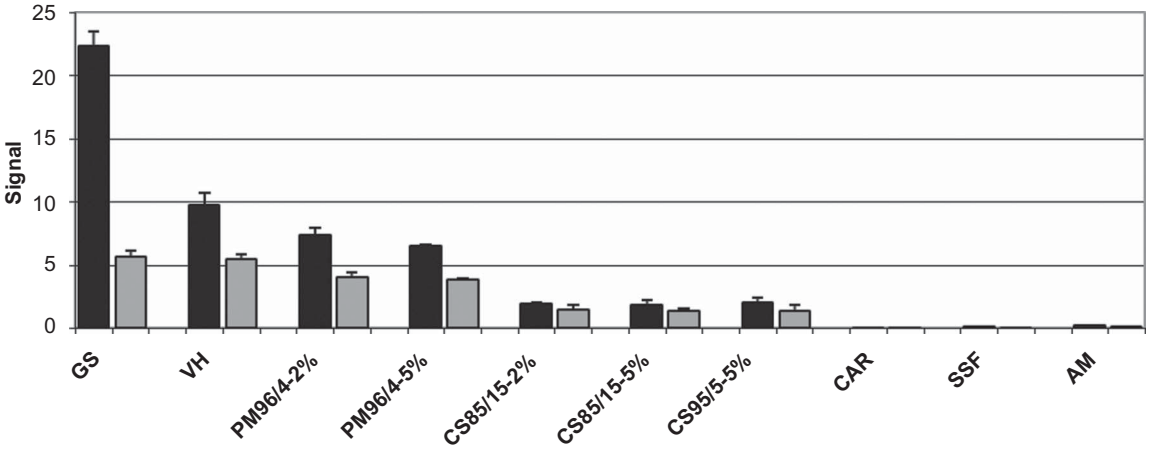

Figure 5. Maximum peak to peak heights of the EPR signals of the radicals (per mg) induced by gamma-irradiation (25 kGy), measured after 1 (■) and 30 (▭) days. GS, gentamicin sulfate; VH, vancomycin hydrochloride; CAR, Carbopol® 974P; SSF, sodium stearyl fumarate; AM, Amioca® starch (mean ± SD, $n = 3$).

Table 4. Drug concentration in the bioadhesive powder mixture after 4 weeks storage (21 ± 3°C) as a function of the gamma-irradiation dose.

	Concentration (%)	
Irradiation dose (kGy)	Vancomycin	Gentamicin
0	–	104.7
10	98.8	99.6
25	99.2	108.3

Results are expressed in percentage of the drug concentration determined immediately after irradiation.

that vancomycin hydrochloride induced severe irritation (Total MP > 20%). In addition, gentamicin sulfate, CS85/15-5%, PM96/4-5%, and CS95/5-5% caused a higher MP compared to the negative controls, resulting in mild irritation of the mucosal surface (total MP between 7% and 12%).

The tissue damaging potential of formulations can be assessed by the protein and enzyme (LDH and ALP) release from the mucosal surface of the slugs. Similar or slightly increased protein release was observed in the first sample after treatment with vancomycin hydrochloride, CS85/15-5%, PM96/4-5%, and CS95/5-5% compared to the negative controls. However, the protein release levels dropped to the basal level in the second sample. None of the powders induced enzyme release. Using the prediction model of Adriaens et al.[34], it can be concluded that none of powder mixtures and drugs tested resulted in tissue damage (Table 5). Because gentamicin sulfate interfered with the NanoOrange™ protein assay, this endpoint could not be used. However, because no enzyme release was detected and the body weight of the slugs after the fifth treatment was still more than 80%, tissue damage is very unlikely. For the positive control slugs the moderate tissue damage resulted in a body weight reduction of almost 50% (Figure 6). Vancomycin hydrochloride induced severe irritation and reduced the body weight to 63% after the fifth treatment, whereas in case of the CS85/15-5%, PM96/4-5%, and CS95/5-5% powder mixtures the body weight was more than 75%.

As the powder mixtures containing gentamicin sulfate and vancomycin hydrochloride are of interest for the improvement of the ocular administration of these drugs, it is important to evaluate their effect on mucosal tissues. Powder mixtures inducing no irritation and no tissue damage are promising for ocular use. The cospray-dried and physical powder mixtures caused only mild irritation, whereas no tissue damage was observed. Test items that are classified as mild irritants with the SMI assay may increase the probability of stinging and burning sensations in humans. Moreover, the minitablets prepared by cospray-dried mixture of Amioca® starch with Carbopol® 974P (95/5) containing 5% (w/w) gentamicin sulfate and 5% (w/w) vancomycin hydrochloride (CS95/5-5% coded formulation) is optimal for further evaluation in volunteers as the slowest release profile has been achieved with this formulation and it caused only mild irritation, whereas no tissue damage was observed.

Conclusion

Ocular bioadhesive minitablets containing gentamicin sulfate and vancomycin and formulated with cospray-dried powder mixtures of starch and Carbopol provided sustained drug delivery and their properties were not affected by gamma irradiation (used a sterilization technique of the ocular dosage form). In addition, this concept is promising for in vivo application as the SMI test only indicated mild irritation properties of these formulations.

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Table 5. Effect of the composition of drug substance and bioadhesive powder mixtures on the endpoints of the slug mucosal irritation test (mean ± SD, $n = 5$).

Formulations	Total MP (%)	Protein (μg/mL.g)	LDH (U/L.g)	ALP (n)	Irritation/tissue damage
DDWM	3.3 ± 1.5	6.7 ± 3.5	–	–	Non-irritant/no
DDWM/SLS (80:20)	35.9 ± 7.7	58.5 ± 9.7	0.28 ± 0.28	3/5	Severe/moderate
Vancomycin HCl	27.2 ± 5.0	16.1 ± 6.6	–	–	Severe/no
Gentamicin sulfate	10.1 ± 0.6	ND	–	–	Mild/no
CS95/5-5%	8.5 ± 1.9	6.7 ± 1.8	–	–	Mild/no
PM96/4-5%	8.9 ± 2.3	13.7 ± 2.8	–	–	Mild/no
CS85/15-5%	9.3 ± 1.8	9.7 ± 2.5	–	–	Mild/no

ND, not determined.

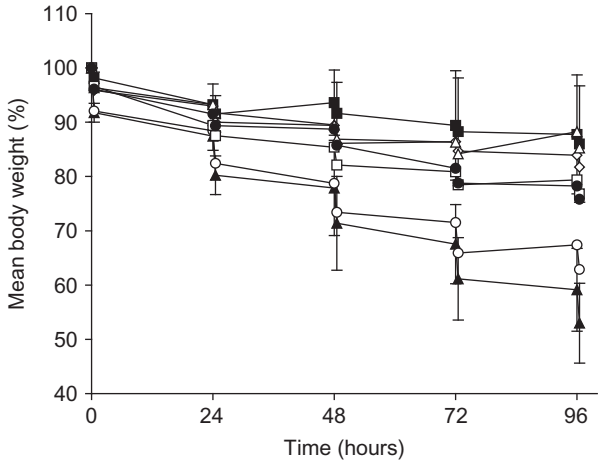

Figure 6. Mean body weights (initial body weight %) of the slugs treated with drug substance or bioadhesive powder mixtures. —■—, DDWM; —▲—, DDWM/SLS (80/20); —○—, vancomycin hydrochloride; —◇—, gentamicin sulfate; —△—, CS95/5-5%; —□—, PM96/4-5%; —◆—, CS85/15-5% (mean ± SD, $n = 5$).

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this paper.

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