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Malonylchitosan Microspheres as a Matrix for Oral Enrofloxacin Delivery

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Summary: The purpose of this study was to produce malonylchitosan microspheres to be used as a pH sensitive matrix for the controlled delivery of drugs. Chitosan microspheres were obtained by the coacervation–phase separation method. The microspheres were then treated with malonic acid to obtain amidified chitosan microspheres. The resulting malonylchitosan microspheres were characterized by means of elemental analysis (CHN), infrared spectroscopy, conductometric titration and scanning electron microscopy (SEM). The data obtained from infrared spectroscopy, elemental analysis and conductometric titration of amino groups were consistent with the N-substitution of malonyl groups in the chitosan microspheres. *In vivo* studies for controlled delivery of enrofloxacin were performed in male Wistar rats and the results indicated increase and prolonged blood levels with delayed peak and improved bioavailability for malonylchitosan microspheres compared to commercial tablets.

Keywords: biopolymers; drug delivery systems; enrofloxacin; *in vivo* studies; malonylchitosan

Introduction

The development of new polymeric materials in the pharmaceutical industry is one such area of increasing interesting in the last years, focusing on the use of microparticles as useful systems employing novel and low cost manufacturing encapsulation techniques to stabilize, protect and mask the undesirable flavors or still to modify the release properties.[1,2]

These microparticle systems have reached a great interest in the oral formulations, since they present advantages over single-unit dosage forms such as low variability in pharmacokinetics in the gastrointestinal tract (GIT), good dispersion, possibility of mixing particles with different release properties and reduction of the number of the doses.[3] One primary approach to protecting both the drug incorporated in the system as well as the system itself from degradation in the gastric juice is to coat the system with biocompatible, nontoxic polymers that are insoluble at acidic pH but swell in the alkaline pH of the intestine and release the drug. Thus, this drug delivery system can be efficient for the treatment of different pathologies.

Chitosan, a biocompatible and biodegradable polymer, with low toxicity, soluble at acidic pH and insoluble at basic pH, has received considerable attention as a valuable pharmaceutical excipient in the form of microspheres to control the release of drugs.[4,5] Modifications in the characteristics of the chitosan microspheres with different compounds as malonic acid can produce derived with high swelling degree at the neutral pH with desired release properties.[6]

Veterinary drugs are defined as any substances used in or on animals to prevent, diagnose, treat or relieve a disease, disorder

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or symptom thereof. The importance of fluoroquinolones as veterinary drugs has steadily increased during the last years. Fluoroquinolones are first-line drugs for empiric therapy of acute diarrhea, as they are effective against most major pathogens causing bacterial enteritis.[7,8] The principal fluoroquinolones developed are the enrofloxacin (Baytril®, Bayer), orbifloxacin (Orbax TM, Schering-Plough Animal Health), difloxacin (Dicural®, Fort Dodge Animal Health) and marbofloxacin (Zeniquin®, Pfizer Animal Health). Others fluoroquinolones, which are used in humans such as ciprofloxacin (Cipro®, Bayer) are also used in veterinary medicine for small animals, such as small dogs and cats.[9]

Enrofloxacin, a synthetic antimicrobial agent from class of fluoroquinolones, has been developed exclusively for use in veterinary medicine.[10,11] This drug has a good antimicrobial activity and a broad spectrum of action against a wide class of gram-positives and gram-negatives bacteria, including those resistant to the β-lactamic and sulfonamide antibiotics.[12] Enrofloxacin is reported to cause side effects, such as gastric discomfort, nauseas, vomits, chronic headache, toxic effects in the central nervous system (CNS), and still presents distasteful flavor making difficult its oral administration in animals. An approach to reduce these side effects after oral administration could be achieved by delivering the enrofloxacin by use of enteric polymers in microspheres matrices based on chitosan.

The purpose of this study was to produce malonylchitosan microspheres by amidification of chitosan microspheres with malonic acid. The amidified chitosan was used as a pH sensitive matrix for the *in vivo* controlled delivery of the veterinary drug enrofloxacin.

Materials and Methods

Materials

The enrofloxacin reference substance (assigned purity 90%) (Baytril®, Bayer) was a gift from Brazil, Sementes Prezzotto Ltda.

Male Wistar rats, weighing 250 ± 25 g, were housed under controlled conditions (12 h light-dark cycle, 22 ± 2 °C, 60% air humidity) and had free access to standard laboratory chow and water. All animals were allowed to acclimatize for at least 5 d prior to the first treatment. Animals were fasted for 12 h before experiments and allowed water *ad libitum*. All animal procedures were conducted in accordance with legal requirements appropriate to the species.

Preparation of Chitosan Microspheres

Fine chemical-grade chitosan, derived from shrimp-shell chitin following about 90% descetylation, was dissolved in acetic acid 5% (m/v) in order to produce a viscous 4% (m/v) chitosan solution and subsequently poured into a bath containing NaOH 2.0 $\text{mol} \cdot \text{L}^{-1}$ solution through a Ismatec peristaltic pump in order to obtain gelled microspheres.[13]

Preparation of Malonylchitosan Microspheres

An aqueous suspension of chitosan microspheres was filtered and the excess water was removed by suction. 5 g of the moist chitosan microspheres were suspended in 20 mL anhydrous ethanol and stirred for 1 h. The ethanol was filtered off and then replaced with a fresh supply of anhydrous ethanol (30 mL). The mixture was stirred for 30 min. This procedure was repeated three times.

The never-dried chitosan microspheres were suspended in 20 mL anhydrous ethanol and 450 mg malonic acid was added to the microsphere suspension. The reaction mixture was refluxed for 36 h at 78 °C and then cooled to room temperature. The modified chitosan microspheres obtained were washed twice with 30 mL portions of ethanol.[14]

The microspheres were then placed in contact with glutaraldehyde 2.5% (m/v) solution for a cross-link period of 12 h at room temperature. The resulting material was washed with distilled water to remove the excess of cross-linking agent.

The cross-linked malonylchitosan microspheres were dried at room temperature. The microspheres were found to be insoluble in dilute acetic acid, neutral pH water, and dilute sodium hydroxide solution. They were also found to swell in aqueous or non-aqueous solvents.

Characterization of Malonylchitosan Microspheres

The chitosan microspheres, which were modified with malonic acid, were characterized by infrared absorption spectroscopy, elemental analysis (CHN), conductometric titration and scanning electron microscopy.

The total primary amino groups content of the chitosan microspheres was determined by conductometric titration carried out under nitrogen atmosphere, using an automatic analyzer model Schott Geräte-T80/20 and a Micronal B330 conductivimeter.

The infrared spectra were collected on a FT-PC-16 model Perkin-Elmer spectrophotometer. Samples were prepared as KBr pellets.

The substitution of malonyl groups in the original chitosan microspheres was confirmed by the increase in the atomic ratio of carbon to nitrogen (C/N) of the malonyl chitosan microspheres ($C/N = 7.77$), compared with that of the original chitosan microspheres ($C/N = 5.30$). The C/N values were measured with an Elemental Analyzer Carlo Erba EA 1 110.

Elemental analysis, Found C 36.67%, H 6.71% and N 4.71% for malonylchitosan microspheres (36 h), and C 38.81%, H 7.4% and N 6.95% for original chitosan microspheres.

In order to study the shape, size and internal and external morphologies of the microspheres, samples were freeze-dried and analyzed by scanning electron microscope (Philips, Model XL 30).

Enrofloxacin Loading on Malonylchitosan Microspheres

The malonylchitosan microspheres were impregnated with enrofloxacin using a contact adsorption technique with best dissolution medium on drug impregnation. Solid enrofloxacin was dissolved in KOH 1 mol $\cdot$ L^{-1} solution and the resulting solution was impregnated by malonylchitosan microspheres for 48 h at room temperature under stirring 100 rpm. After neutralizing with HCl 1 mol $\cdot$ L^{-1} solution and removing the excess enrofloxacin with distilled water, the malonylchitosan microspheres were dried at room temperature. The concentration of the drug impregnated into the malonylchitosan microspheres was determined by UV-Vis spectrophotometry at 276 nm from the dissolution of enrofloxacin in KOH 1 mol $\cdot$ L^{-1} solution. The efficiency of impregnation was of the 520 mg of enrofloxacin per gram of dried malonylchitosan microspheres.

In vivo Drug Release Studies

Male Wistar rats weighing 200–250 g were housed under controlled conditions (12 h light-dark cycle, 22 ± 2 °C, 60% air humidity). Rats were randomly divided into groups of six and were fed with rodent chow and tap water *ad libitum*. Enrofloxacin microspheres or enrofloxacin not encapsulated were dissolved in distilled water, and administered via oral in dose of 5 mg $\cdot$ kg^{-1} body weight (near to veterinary oral therapeutic doses).[15,16] Controls received only microspheres without drug under the same conditions. Blood was collected by ocular puncture in anaesthetized animals in 30 min, 1, 3, 6, 9, 12, 24 h in accordance with the principles set out in the *"International Guide to the Care and Use of Experimental Animals"* appropriate to the species and centrigugated at 4 000 rpm for 5 min. Serum was separated to determination of enrofloxacin concentration by spectrophotometry UV in 276 nm.[17]

Results and Discussion

Amidification Reaction

The use of anhydrous ethanol in the preparation of malonylchitosan was necessary since the presence of water is undesired during the amidification reaction as it slows down the reaction and lowers the degree of substitution to be attained. The dicarboxylic groups of malonic acid, when reacting with amino groups of chitosan under heating, results in an inter-molecular reaction by removing water with one equivalent of amine to form a malonylchitosan material.[14] The amidified chitosan was characterized by infrared spectroscopy, elemental analysis (CHN), conductometric titration and scanning electron microscopy (SEM).

Infrared Analysis

The infrared spectra of malonylchitosan microspheres are shown in Figure 1. The absorption bands at 3 424–3 446 cm^{-1} of modified microspheres are rather intense as a consequence of OH and water stretching vibrations. The band at 1 377 cm^{-1} also observed for the original chitosan microspheres can be attributed to deformation vibrations of medium intensity of N–H bonds from primary amines and of low intensity from C–H bonds of the CH_3 group. Small amounts of residual acetamide groups can be found in the polymeric chain as a consequence of incomplete deacetylation of chitosan. The band at 1 072 cm^{-1} corresponds to the vibrational stretching of C–O bonds from primary alcohol.

The absorption band at 1 636 cm^{-1} observed for the modified chitosan corresponds to the vibrational deformation of N–H (amide II), which is absent in the original chitosan microspheres. The absorption band at 1 721 cm^{-1} is assigned to aliphatic carboxylic acid, which is not observed in the original chitosan. This suggests the formation of a monoamide group and a monocarboxylic acid group after 36 h for the resulting malonyl derivative.

Conductometric Titration and Elemental Analysis

The percentage of free amino groups in malonylchitosan (36 h) was verified by conductometric titration with NaOH 0.10 mol · L^{-1} solution. The amino groups content found was 11.0% which corresponds to 80% of substituition by considering that original chitosan was 90% deacetilated.

The degree of substitution (DS) of malonyl groups in the original chitosan microspheres was also calculated according to the method described by Inukai and coworkers as follows:[18]

$$DS = [(C/N)_r - (C/N)_o]/3 \qquad (1)$$

where $(C/N)_r$ is the C/N ratio of the resulting malonylchitosan microspheres,

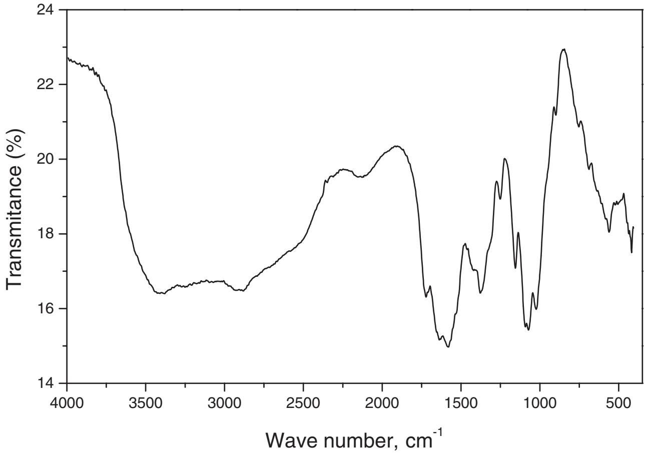

Figure 1.
Infrared spectrum of malonylchitosan obtained after 36 h.

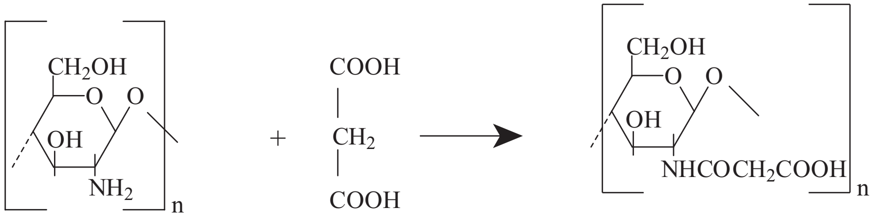

Figure 2.
Structure of chemically modified chitosan with malonic acid.

and $(C/N)_o$ is the C/N ratio of the original chitosan microspheres and 3 is the number of carbons after chitosan modification.

The DS of the resulting malonylchitosan microspheres was 82%. Therefore, it is reasonable to consider that an N-substitution by the malonyl groups is occurring given that the substituted amino groups content determined by conductometric titration on modified chitosan microspheres before cross-linking was 80%, supporting the previous elemental analysis results. The substitution by malonyl groups in the original chitosan microspheres can occur for both the more reactive hydroxy group at C6 and the amino group at C2 of chitosan. However, N-substitution is reported to be preferred over O-substitution.[18]

The characterization studies carried out through infrared and elemental analysis proved the amidification of chitosan, and the probable structure of amidified product is shown in Figure 2.

Swelling Degree (SD)

Swelling degree (SD) is an important parameter associated with the mechanism and kinetics of hydrogels.[19]

The Swelling degree (SD) in percent was calculated in this work by Equation (2), where W_1 and W_2 represent the weight of dry cross-linked malonylchitosan microspheres and the weight of wet cross-linked malonylchitosan microspheres, respectively.

$$\%SD = (W_2 - W_1)/W_1 \times 100 \qquad (2)$$

The SD of 188% was obtained from the weight of malonylchitosan microspheres determined after removing the surface water by blotting with tissue paper and the dry weight after dehydration under vacuum at room temperature to constant weight.

The swelling properties of ionic hydrogels are unique due to ionization of their pendent functional groups. Physiological parameters such as pH can change drastically the equilibrium degree of swelling by several orders of magnitude.[20] Therefore, the cross-linked malonylchitosan microspheres prepared here, behaved as polyelectrolyte hydrogels, and may be used as carriers for swelling-controlled enrofloxacin delivery systems. The change in pH in the gastrointestinal tract acts as a release stimulus for the hydrogel. Ionic hydrogels can be used as drug carriers in oral dosage forms to deliver drugs selectively to the intestine.

Scanning Electron Microscopy (SEM)

Typical electron micrographs of malonylchitosan microspheres are shown in Figure 3. Electronic microscopies of the samples revealed a mixed population of microspheres, some having good sphericity, presenting a smooth surface with apparent porosity. The average particle size for these microspheres was 1.05 ± 0.5 [Figure 3(a)].

The SEM micrograph of the cross-sections of microspheres [Figure 3(b) and (c)] revealed a monolithic porous internal structure.

In vivo Enrofloxacin Release from Malonilchitosan Microspheres

In vivo evaluation of the enrofloxacin release from malonylchitosan microspheres was carried out in healthy, normal rats by

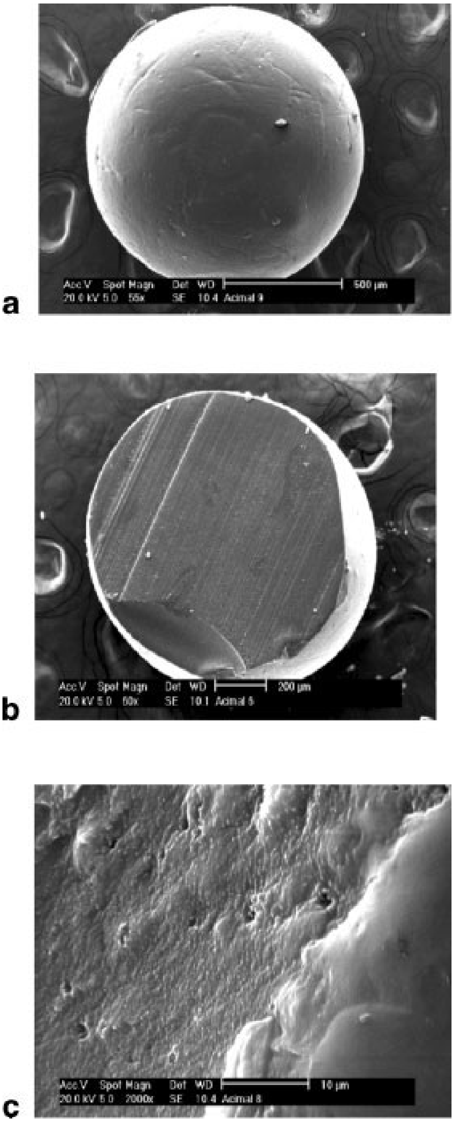

Figure 3.
SEM micrographs cross-section of (a) malonylchitosan microsphere at lower magnification (55×), (b) cross-section of a malonylchitosan microsphere at lower magnification (60×) and (c) cross-section of a malonylchitosan microsphere at higher magnification (2 000×).

measuring the spectrophotometric serum levels of free drug after oral administration at a dose equivalent to 520 mg · kg^{-1} of impregnated enrofloxacin, in comparison to free enrofloxacin at the same dose. The results from these studies are shown in Figure 4. Oral administration of free enrofloxacin (F-ER) resulted in a maximum peak concentration of 488 μg · ml^{-1} in 1 h and this drug level was sustained over 3 h. A non-exponential serum concentration decline after this time in accord with data obtained by Papich et al.[9] and Otero et al.[10] However, in the case of malonylchitosan microspheres containing drug (MLQT-ER), there was an increased serum level of enrofloxacin with a maximum peak around 670 μg · ml^{-1}. It was maintained during the period from 6 to 12 h. The increase and sustained effect observed over longer period of time in the case of microspheres is probably due to the slow release and absorption of enrofloxacin over longer periods of time. Therefore, the microencapsulation of enrofloxacin represent a change in kinetic parameter and an increase in blood drug concentration what could be an advantage to veterinary therapeutic use of this antimicrobial drug.

Conclusion

The absorption bands assigned to amide (II) and aliphatic carboxylic acid, which are not observed in the original chitosan, suggest the formation of a monoamide group and a monocarboxylic acid group for the resulting malonyl derivative.

The N-substitution by the malonyl groups was determined by conductometric titration on modified chitosan microspheres before cross-linking was 80%, supporting the previous elemental analysis results of 82%.

The malonylchitosan presented hydrogel behavior with high swelling degree, which may be used as carrier for swelling-controlled drug delivery systems.

The results of the *in vivo* studies indicated increase and prolonged blood levels with delayed peak and improved bioavailability for malonylchitosan microspheres compared to commercial tablets.

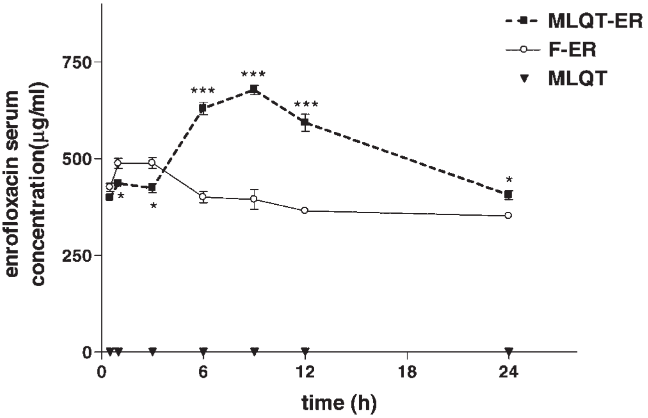

Figure 4.
In vivo delivery of the malonylchitosan microspheres with impregnated enrofloxacin (MLQT-ER); free enrofloxacin (F-ER) and the malonylchitosan microspheres (MLQT) in rats dealt with 520 mg · kg^{-1} (v.o). All values are expressed as mean ± SEM, $n = 6$, (*) and (***) denotes significant difference in relation to F-ER ($P < 0.001$ and $P < 0.05$).

The malonylchitosan microspheres reported here can have a significant application to drug delivery systems where release is a pH sensitive function.

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[1] I. Kazuhiro, M. Yoshiharu, S. Takanori, N. B. Tsunejl, *Drug Des. Deliv.* **1989**, *4*, 55.
[2] I. Kazuhiro, M. Yoshiharu, S. Takanori, N. B. Tsunejl, *Drug Des. Deliv.* **1989**, *1*, 297.
[3] A. T. Florence, P. U. Jani, *Drugs Safety* **1994**, *19*, 233.
[4] K. Amano, E. Ito, *Eur. J. Biochem.* **1978**, *85*, 97.
[5] F. Nakamura, H. Onischi, Y. Machida, *Yakuzaigaku* **1992**, *52*, 59.
[6] S. A. Brown, *J. Vet. Pharmacol. Ther.* **1996**, *19*, 1.
[7] W. Graninger, K. Zedtwitz-Liebenstein, H. Laferl, H. Burgmann, *Chemotherapy* **1996**, *42*(*Suppl. 1*), 43.
[8] J. Wistrom, S. R. Norrby, *J. Antimicrob. Chemother.* **1995**, *36*(*Suppl 1*), 23.
[9] M. G. Papich, S. D. Van Camp, J. A. Cole, M. D. Whitacre, *J. Vet. Pharmacol. Ther.* **2002**, *25*(*5*), 343.
[10] J. L. Otero, N. Mestorino, J. O. Errecable, *Analecta Veterinaria, Part I* **2001**, *21*(*1*), 31.
[11] J. L. Otero, N. Mestorino, J. O. Errecable, *Analecta Veterinaria, Part II* **2001**, *21*(*1*), 42.
[12] M. Scheer, *Vet. Med. Rev.* **1987**, *2*, 104.
[13] G. L. Rorrer, T. Hsien, J. D. Way, *Ind. Eng. Chem. Res.* **1993**, *32*, 2170.
[14] US 5,874,551 (**1999**), Center for Innovative Technology, invs.: W. G. Glasser, R. K. Jain.
[15] J. S. Wolfson, D. C. Hooper, *Eur. J. Clin. Microbiol. Infect. Dis.* **1991**, *10*, 267.
[16] R. M. Linnehan, R. W. Ulrich, S. Ridgway, *J. Vet. Pharmacol. Theor.* **1999**, *22*, 170.
[17] M. Lizondo, M. P. Gallardo, M. J. Estelrich, *J. Pharm. Biomed. Anal.* **1997**, *15*, 1845.
[18] Y. Inukai, T. Chinen, T. Matsuda, Y. Kaida, S. Yasuda, *Anal. Chimc. Acta.* **1998**, *18*(*5*), 679.
[19] I. Y. Kim, S. J. Kim, Y. M. Lee, D.-I. Shin, S. I. Kim, *J. Appl. Polym. Sci.* **2002**, *85*, 2661.
[20] N. A. Peppas, A. R. Khare, *Adv. Drug Delivery Rev.* **1993**, *11*, 1.