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Mucoadhesive vaginal tablets as veterinary delivery system for the controlled release of an antimicrobial drug, acriflavine

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ABSTRACT The aim of the study was the development of mucoadhesive vaginal tablets designed for the local controlled release of acriflavine, an antimicrobial drug used as a model. The tablets were prepared using drug-loaded chitosan microspheres and additional excipients (methylcellulose, sodium alginate, sodium carboxymethylcellulose, or Carbopol 974). The microspheres were prepared by a spray-drying method, using the drug to polymer weight ratios 1:1 and 1:2 and were characterized in terms of morphology, encapsulation efficiency, and in vitro release behavior, as MIC (Minimum Inhibitory Concentration), MBC (Minimum Bacterial Concentration), and killing time (KT). The tablets were prepared by direct compression, characterized by in vitro drug release and in vitro mucoadhesive tests. The microparticles have sizes of 4 to 12 µm; the mean encapsulation yields are about 90%. Acriflavine, encapsulated into the polymer, maintains its antibacterial activity; killing time of the encapsulated drug is similar to that of the free drug. In vitro release profiles of tablets show differences depending on the excipient used. In particular Carbopol 974, which is highly cross-linked, is able to determine a drug-controlled release from the matrix tablets for more than 8 hours. The in vitro adhesion tests, carried out on the same formulation, show a good adhesive behavior. The formulation containing microspheres with drug to polymer weight ratios of 1:1 and Carbopol 974 is characterized by the best release behavior and shows good mucoadhesive properties. These preliminary data indicate that this formulation can be proposed as a mucoadhesive vaginal delivery system for the controlled release of acriflavine.

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INTRODUCTION In the veterinary field, the development of new drug delivery systems is of considerable interest. The use of controlled-release dosage forms offers numerous benefits including reducing animal stress resulting from restraint, handling, and dosing; and avoiding expensive or difficult drug administration procedures.

The major areas of application of controlled-release technology in the veterinary field are disease prevention and control via delivery of anthelmintics, antibiotics, antiparasitic agents, growth promotions, nutritional agents, and vitamins to companion and food-producing animals [1].

In particular, the intravaginal route has been traditionally used for the delivery of locally acting drugs such as antimicrobial and antiparasitic agents [2]. However, conventional vaginal delivery systems—such as creams, foams, pessaries, and jellies—reside for relatively short periods of time at the targeted site because of the self-cleaning action of the vaginal tract [3,4]. Effective drug levels are consequently limited to a short time period. Therefore, repeated administrations are often required. A vaginal delivery system should provide controlled and prolonged release of drugs.

Many drug delivery systems are based on mucoadhesive polymers [5]. Mucoadhesive delivery systems have been developed both for the local and the systemic administration of drugs through different mucosal routes: buccal [6-8], nasal [9,10], and vaginal [11-14].

The development of a mucoadhesive vaginal dosage form permits the drug to maintain a certain level locally,

Table 1. Compositions, Drug Contents, Encapsulation Efficiencies, and Particle Sizes of Microspheres Prepared*

Formulation	Drug-to-Polymer Weight Ratio	Theoretical Drug Content (%)	Actual Drug Content (%)	Encapsulation Efficiency (%)	Particle Size (μm) (d_w) ± SD
Acri 1	1 : 1	50.0	43.5	86.8	12.570 ± 1.890
Acri 2	1 : 2	33.5	30.3	90.4	6.915 ± 0.089
Acri 0	0 : 1	0	0	0	4.153 ± 0.159

*SD indicates standard deviation.

to extend drug residence time at the administration site, and to reduce dosing frequency and the amount of drug administered [15]. Therefore, a mucoadhesive vaginal delivery system represents a good alternative to the numerous applications typical of conventional dosage forms.

The aim of the present study was the development of a veterinary drug delivery system based on chitosan vaginal tablets. Acriflavine was chosen as a model drug for the following reasons: it is a highly water-soluble drug (1 g dissolves in about 3 mL of water) [16] ; it has a bacteriostatic activity against Gram-positive bacteria; and it is effective against bovine Trichomoniasis [17].

Chitosan is a polysaccharide obtained by the partial deacetilation of chitin. It owns biocompatibility and biodegradability [18] and is widely used as a pharmaceutical excipient [19]. Chitosan also exhibits antimicrobial and wound-healing properties [20,21]. The use of chitosan as mucoadhesive polymer for the human buccal drug administration has already been studied [6].

The preparation of the tablets was carried out by direct compression. To improve the flow properties of chitosan, before the compression, drug-loaded chitosan microspheres were prepared using a spray-drying method and characterized in terms of in vitro release behavior and antimicrobial activity. For the preparation of the tablets, methylcellulose and several mucoadhesive polymers such as sodium alginate, sodium carboxymethylcellulose, or Carbopol 974 were used as additional excipients.

The tablets were tested for their in vitro release behavior and mucoadhesive properties.

MATERIALS AND METHODS

Acriflavine (euflavine) hydrochloride and sodium alginate, high viscosity (2.0% wt/vol aqueous solution at 25°C with a viscosity of approximately 14 000 cps (centipoises) [manufacturer value], were purchased from Sigma Chemical Co (St Louis, Mo). Chitosan, deacetylation degree 75% to 85%, viscosity 200 to 800 cps (1% solution in acetic acid) [manufacturer value], was supplied by Sigma-Aldrich s.r.l. (Milano, Italy). Carbopol 974 was purchased from BFGoodrich (Brussels, Belgium). Metolose (methylcellulose), 90SM, viscosity 15 000 cps, was supplied by Shin-Etsu Chemical (Tokyo, Japan). Sodium carboxymethyl cellulose was purchased from Cruciani Alberto Prodotti Crual (Milano, Italy). Porcine gastric mucin type II (Sigma Chimica, Milan, Italy) was used as biological substrate in mucoadhesion measurements.

Preparation of microspheres by spray-drying

The microspheres were prepared with the drug to polymer weight ratios of 1:1 and 1:2, using a spray-drying technique. Chitosan was dissolved in hydrochloric acid (0.1 M), which was then evaporated to dryness. The residual and the drug (2% total wt/vol concentration; chitosan concentration 1% and 1.33% in 1:1 and 1:2 preparations, respectively) were dissolved in distilled water, producing a solution that was sprayed through the nozzle of a spray-dryer, model Mini Spray HO (Pabisch SpA, Milano, Italy), cocurrent flow type, equipped with a standard 0.7-mm nozzle. The process conditions were the following: inlet air temperature, 98°C-100°C; outlet air temperature, 75°C-76°C; feed rate, approximately about 8 mL · min^{-1}. The spray-dried microparticles were harvested from the apparatus collector and kept under vacuum for 24 hours, at room temperature.
An aqueous solution of chitosan (2% wt/vol) was sprayed to obtain drug empty microspheres (Acri 0), prepared as a comparison. Each preparation was carried out in triplicate. The total amount of solid material used for the preparation of each batch of microparticles was 10 g. **Table 1** reports the compositions of all types of microspheres prepared.

Scanning electron microscopy

The morphology (shape and surface characteristics) of the spray-dried microspheres was studied by Scanning Electron Microscopy (SEM), model DSM 962 (Carl Zeiss Inc, Germany). Samples of microspheres were placed on a double-sided tape, which had previously been secured on aluminium stubs. The samples were then

Table 2. MIC (MBC) Values in µg · mL^{-1} .*

Bacteria	Acriflavine	Acri 1	Acri 2	Acri 0
E coli	39 (78)	90 (90)	180 (180)	1000 (>1000)
Ps aeruginosa	156 (624)	180 (719)	359 (1437)	>1000 (>1000)
S aureus	19 (19)	23 (46)	45 (180)	208 (1000)

*MIC indicates Minimum Inihibitory Concentration; MBC, Minimum Bactericidal Concentration

Table 3. Killing Time (Percentage Survived of E coli)

Time	Acriflavine, %	Acri 1, %	Acri 2, %
after 10 minutes	21.1	15.1	32.6
after 20 minutes	0.5	0.14	1.5
after 30 minutes	0.0	0.0	0.0

analyzed at 20 kV acceleration voltage after gold sputtering, under an argon atmosphere.

Particle size analyses

Particles were sized by the light diffraction method using a Coulter apparatus, model LS 100Q (Beckman Coulter Particle Characterisation, Miami, FL). Particle size analyses were carried out on blank and drug-loaded spray-dried microspheres that were suspended in silicon oil (Tegiloxan3, Goldschmidt, Essen, Germany) and sonicated for about 20 seconds. Three analyses were performed for each sample of microspheres. The average particle size of each sample was expressed as the volume-surface diameter (dvs, µm) (**Table 1**) [22].

Drug content determination

The determination of the acriflavine content was carried out on each batch of drug-loaded microspheres as follows: an amount of microspheres, equivalent to 10 mg (approx) of the drug, was dissolved in 25 mL of distilled water, in a volumetric flask. The solution, after suitable dilution, was measured spectrophotometrically at 262 nm, 1-cm cell (spectrophotometer, model U-2001, Hitachi Instruments, Tokyo, Japan). The polymer did not interfere with the absorbance of the drug at the specified wavelength. The drug content was calculated as a percentage with respect to the theoretical amount of acriflavine and expressed as the mean of 3 replicates for each batch.

Drug contents and encapsulation efficiencies are reported in **Table 1**.

Microbiological assays: minimum inhibitory concentration, minimum bactericidal concentration, killing time

The evaluation of antimicrobial activity was performed on acriflavine (as pure drug), acriflavine-loaded microspheres, and drug-empty microparticles. Minimum Inihibitory Concentration (MIC, µg · mL^{-1}) and Minimum Bactericidal Concentration (MBC, µg · mL^{-1}) were determined by using a broth dilution technique [23 against the following selected microbial strains: Escherichia coli ATCC 25922, Pseudomonas aeruginosa ATCC 27853, and Staphylococcus aureus ATCC 25922. Briefly, 2-fold serial dilutions of a solution of acriflavine pure drug (stock solution:100 mg · mL^{-1} in water) or of microsphere suspensions (1.44 mg · mL^{-1} to 0.023 mg · mL^{-1} in Mueller-Hinton Broth (Oxoid Ltd, Hampshire, England) were prepared in triplicate and inoculated with 5 x 10^5 to 1 x 10^6 organisms mL^{-1}. Test tubes were optically checked after 24 hours of incubation at 35°C, and MICs were defined as the lowest concentration that completely inhibited bacterial growth. Suspensions from tubes not showing bacterial growth were subcultured onto Mueller-Hinton Agar (Oxoid) plates and incubated at 35°C. MCBs were recorded, after overnight incubation at 35°C, as the lowest concentration at which no growth was detectable. MIC and MBC values (µg · mL^{-1}) are reported in **Table 2**.

Killing Time (KT) was determined as the exposure time required to kill a standardized microbial inoculum. E coli in the logarithmic phase of growth were suspended at a density of 5 x 10^{-5} to 1 x 10^6 cfu (colony-forming units) · mL^{-1} in 10 mL of phosphate buffer (pH 7.3) containing appropriate concentrations of acriflavine (as a solution) or Acri 1 and Acri 2

Table 4. Composition of Tablets (100 mg Weight) Prepared from Drug-Loaded Microspheres

Tablets	Microspheres With Drug to Polymer Ratio	Excipient
A	1:1	methylcellulose
B	1:1	sodium carboxymethylcellulose
C	1:1	sodium alginate
D	1:1	Carbopol 974
E	1:2	Carbopol 974

microspheres (as suspensions). The concentrations used were 78 µg · mL^{-1}, 180 µg · mL^{-1}, and 260 µg · mL^{-1} respectively. The concentration of drug used corresponded to its MBC against E coli. A quantity of microspheres corresponding to the same drug concentration was used. A control tube (bacteria suspended in phosphate buffer at the same density) was included in each assay. At regular intervals, 0.5 mL of suspension was removed, diluted in phosphate buffer, and seeded on Tryptone Soya Agar (Oxoid) plates. The number of viable bacteria at each time was evaluated, counting colonies after incubation for 24 hours at 35°C. KT values (in minutes) are reported in **Table 3**.

Preparation of the tablets

Table 4 reports the composition of the tablets prepared from drug-loaded microspheres and the excipients. Formulations A-D were obtained by direct compression of 20 mg of Acri 1 microspheres and 80 mg of each of the following excipients: methylcellulose, sodium alginate, sodium carboxymethyl cellulose, or Carbopol 974. Acri 2 microparticles (20 mg) were compressed with Carbopol 974 into tablets E. In all cases, the powders were mixed in a Turbula apparatus (WA Bachofen, Basel, Switzerland), for 10 minutes at 30 rpm, and then compressed using a hydraulic press equipped with 13-mm flat-faced punches, at the compression force of 1000 kg (hardness of the tablets about 8 kP).

As a comparison, a tablet formulation was prepared using the physical mixture of chitosan, acriflavine, and Carbopol 974, using the conditions reported above (formulation F).

The total weight of each tablet was 100 mg, corresponding to 10 mg (approx) of drug for A-D and F, and to 6 mg (approx) for formulation E.

In vitro release studies

The in vitro release tests were performed using an autosampler dissolution tester, Erweka DT70 (Erweka GmbH, Heusenstamm, Germany). The drug release from the microspheres was determined and compared with the dissolution behavior of acriflavine alone, using the USP 24 apparatus n 2.

The tests were carried out as follows: an amount of microspheres corresponding to 9 mg of drug or about 9 mg of drug was suspended in 1000 mL of USP phosphate buffer (pH 7.4, 100 rpm, 37°C). At each time point, 1 mL of solution was collected and the drug concentration determined spectrophotometrically at 262 nm using Hitachi spectrophotometer, model U-2001 (Hitachi Instruments, Tokyo, Japan); 1 mL of fresh buffer was replaced in the dissolution medium.

The in vitro release tests of the tablets were carried out using a USP dissolution test apparatus n 2 and 1000 mL of phosphate buffer (pH 7.4, 100 rpm, 37°C). Samples of 1 mL were withdrawn at different time intervals up to 24 hours and assayed spectrophotometrically at 262 nm. The initial volume of the medium in the vessel was maintained constant by adding 1 mL of buffer after each sampling. The results obtained in all the tests are reported as mean of 3 determinations (SD within about 3%).

Mucoadhesion measurements

Mucoadhesion measurements were performed by means of a tensile tester previously described [24]. Each tablet was glued on a filter paper disc, which was fixed with a double-sided adhesive tape to the sample holder; the tablet was thereby hydrated with 100 µL of 0.05 M KH_2PO_4 /Na_2HPO_4 buffer pH 5.0, for 5 minutes. Another filter paper was fixed, facing the tablet, on the movable carriage of the tensile apparatus, and it was wetted with either 100 µL of pH 5.0 buffer (blanks) or with 100 µL of 8% (wt/wt) mucin solution in the same buffer. The carriage was moved to place the filter paper and the tablet in contact, and a preload of 2000 mN was applied for 3 minutes. The carriage was then moved away at a constant speed of 4 mm · min^{-1} up to complete separation of the 2 surfaces. Both displacement and force of detachment were recorded on a personal computer.

Force versus displacement curves were analyzed to evaluate the maximum force of detachment (Fmax) and to calculate the work of adhesion as the area under the curve (AUC) by means of the trapezoidal rule.

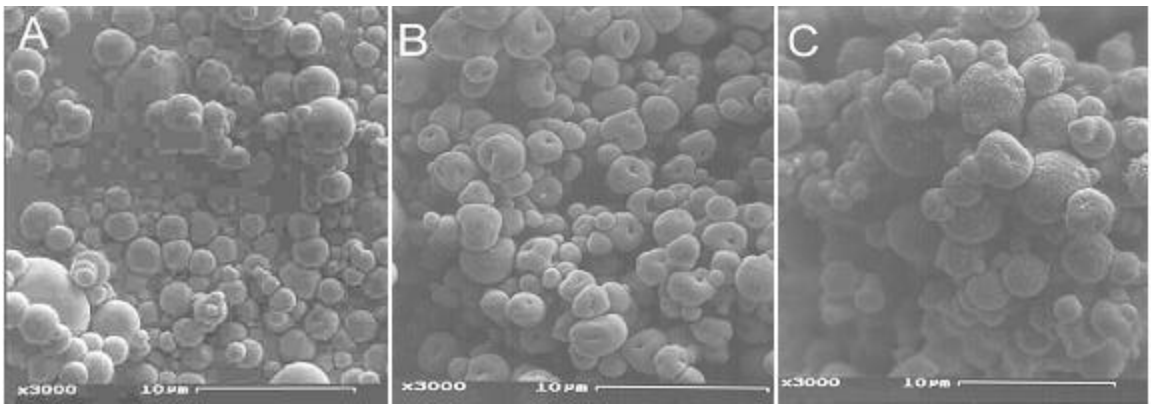

Figure 1A-C. Scanning electron micrographs of microspheres Acri 0 (A), Acri 1 (B), and Acri 2 (C). Photographs were taken at 20 kV.

RESULTS AND DISCUSSION Spray-drying is a good technique for the preparation of chitosan microparticles: it is a 1-step process that involves the preparation of a solution containing drug and polymer.

The SEM images of spray-dried chitosan microspheres (**Figures 1a-c**) reveal that drug-empty microparticles (Acri 0) are characterized by spherical shape and smooth surface. Microspheres with the highest drug load (Acri 1) have irregular shape and are characterized by numerous invaginations. Acri 2 microspheres (drug-to-polymer weight ratio 1:2) have a spherical shape, a quite rough surface, and are free from structural defects. In all cases no free drug crystals appear.

The results of particle size analyses are reported in **Table 1**. Drug-empty microspheres are characterized by the lowest size: dvs about 4 µm. Drug-loaded particles Acri 1 and Acri 2 have a dvs of 12.6 and 6.9 µm, respectively. Granulometric analyses suggest that microsphere size increases with increased drug loading.

As reported in **Table 1**, the spray-drying process permits the load into the polymer amounts of drug close to the theoretical values. The actual drug content is approximately 33% for the microspheres with drug-to-polymer ratio of 1:1 and 18% for the particles with the drug-to-polymer ratio of 1:2. The mean encapsulation yields are 87% and 91%, respectively.

The results of the microbiological tests expressed as MIC and MBC ($\mu g \cdot mL^{-1}$) are reported in **Table 2**. Against the microbial strains, acriflavine as pure drug exhibits MIC values that range between 19 and 156 $\mu g \cdot mL^{-1}$ and MBC values that range between 19 and 624 $\mu g \cdot mL^{-1}$. Against S aureus MIC and MBC values are superimposed (19 $\mu g \cdot mL^{-1}$ in both cases). Drug-empty microparticles (spray-dried chitosan) have a mild antimicrobial effect, because of the polymer itself, confirming previous studies [6]. Drug-loaded microspheres always have higher MIC and MBC values with respect to acriflavine pure drug. However, considering the actual drug contents, the drug activity in the loaded particles is always maintained or improved. For example, acriflavine MIC against S aureus is 19 $\mu g \cdot mL^{-1}$, while Acri 1 microspheres (drug content of 43.4%) exhibit a MIC of 23 $\mu g \cdot mL^{-1}$, which corresponds to 9.98 $\mu g \cdot mL^{-1}$ of acriflavine. Similarly, MIC of Acri 2 is 45 $\mu g \cdot mL^{-1}$ corresponding to 13.6 $\mu g \cdot mL^{-1}$ of pure drug. KT values (in minutes) are reported in **Table 3**. The evaluation of contact times needed to kill an appropriate inoculum shows that the loading of acriflavine in the polymeric microparticles does not lead to remarkable changes with respect to the behavior of acriflavine (pure drug). In fact acriflavine, both alone and encapsulated in the chitosan microparticles requires short exposure time to carry on its bactericidal effect. In particular, after 10 minutes of contact, Acri 1 and Acri 2 microparticles allow the recovery of 15.1% and 32.6% of survived E coli respectively, in comparison with 21.1% of acriflavine as pure drug. After 30 minutes, the percentage of survivors is 0% in all 3 cases.

Figure 2 reports the in vitro release profiles obtained from the drug-loaded microspheres, compared with the dissolution profile of the pure drug. Both batches of microspheres are not able to modulate the acriflavine release in comparison with the dissolution behavior of the pure drug. In fact the in vitro release profiles of microparticles are almost superimposed to the dissolution profile of the drug alone. This behavior could be due to the high solubility of the drug (which is a hydrochloride) in aqueous media [16].

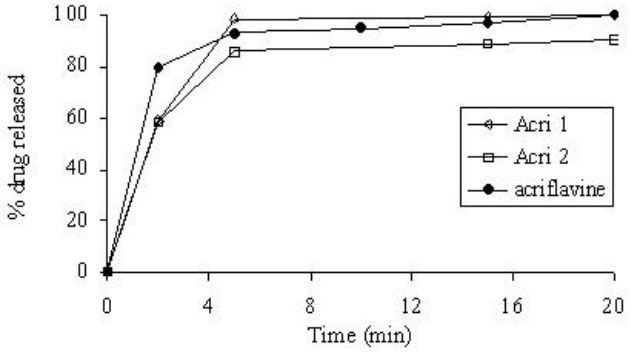

Figure 2. In vitro release profiles of drug-loaded microspheres, compared with the dissolution profile of acriflavine (pure drug).

The spray-dried microspheres mixed with a suitable excipient can be easily tabletted by direct compression, as they are characterized by good flow and compaction properties. **Figure 3** shows the in vitro release patterns of acriflavine from tablets A-D, obtained by compression of Acri 1 microspheres and the different polymers used as additional excipients. The in vitro release properties of the matrices are affected by the excipient used. Formulation A, containing methylcellulose, releases the total drug amount within 30 minutes. Thus, the addition of methylcellulose, which is a nonionic, to the chitosan microspheres determines almost no control of drug release, similar to microparticles alone. On the contrary, formulations B-D are able to control acriflavine release, even if for different times: tablets containing Carbopol 974 release almost 100% of the drug within 8 hours, formulations based on sodium alginate and sodium carboxymethylcellulose furnish a complete drug release within 6 and 3.5 hours, respectively.

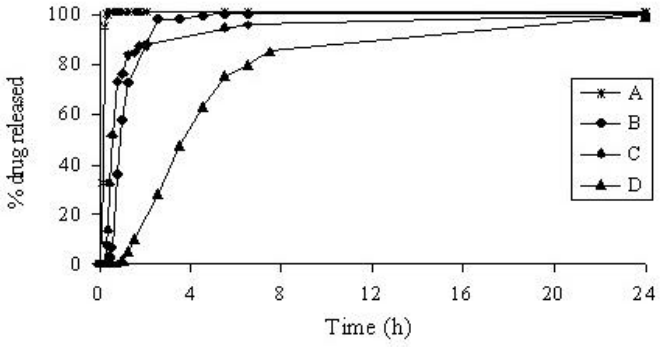

Figure 3. In vitro release profiles of tablets containing Acri 1 microspheres.

These in vitro results could be due to possible ionic interactions among chitosan, a cationic polymer used for the preparation of the microspheres, and the anionic polymers. In fact, it is already known that the cationic nature of chitosan permits the formation of complexes with oppositely charged drugs and/or polymers [25]. Furthermore, it is known that chitosan reacts with polyanions, such as sodium alginate, resulting in a gel [19]. Moreover, Carbopol 974 is a highly cross-linked polymer that swells in water. Because of this structure, the drug dissolution rate from formulation D is delayed in comparison with the dissolution rate of the tablets based on linear polymers, such as sodium alginate and sodium carboxymethylcellulose. Tablets containing Carbopol 974 swell remarkably in the dissolution medium and do not disintegrate up to 24 hours.

Starting from these preliminary results, the other 2 formulations, E and F, were prepared using the same composition as tablet D but containing either Acri 2 microspheres (E) or mixtures of free drug and chitosan (F) instead of Acri 1. Their in vitro drug-release profiles are reported in **Figure 4**. All matrices show extended drug-release behavior. However, formulations containing chitosan as microparticles control the acriflavine release better than formulation F, which uses tabletted chitosan as free powder.

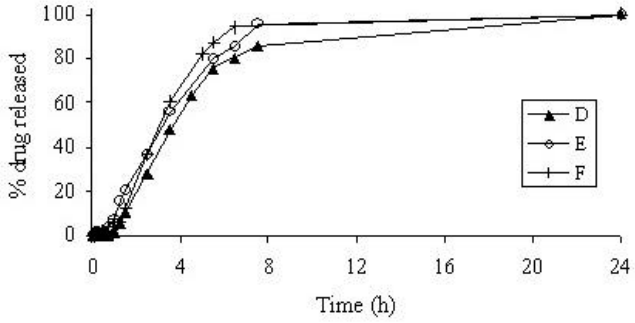

Figure 4. In vitro release profiles of tablets containing Carbopol 974 as additional excipient.

The formulation D, characterized by the best release behavior, has been chosen for the in vitro mucoadhesive study. The data obtained from this test are plotted in **Figure 5**. They show that for both the parameters involved in the study, the maximum force of the detachment (Fmax) and the work of adhesion (AUC), the values obtained in the presence of mucin are remarkably and significantly higher than in the case of the blanks, indicating a good adhesion property of the formulation.

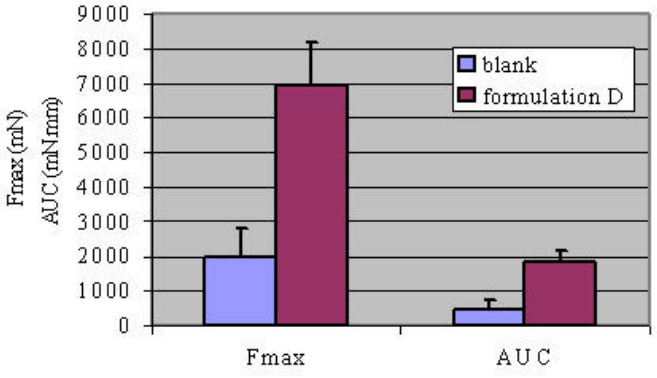

Figure 5. In vitro adhesive tests: maximum force of detachment (Fmax) and work of adhesion, area under the curve (AUC).

CONCLUSION This research shows that spray-drying is a good preparation method of acriflavine-loaded chitosan microspheres. The microparticles can be easily compressed into matrix tablets, combined with suitable excipients. Among the excipients studied, Carbopol 974 was able to give the best results in terms of release behavior. This formulation (D) was studied with in vitro mucoadhesion tests, showing good mucoadhesive properties. These preliminary results indicate the potential use of the proposed formulation as veterinary mucoadhesive vaginal dosage form.

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