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Hyaluronan-based microspheres as tools for drug delivery: a comparative study

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Abstract

The present paper describes the production of biodegradable microparticles using different hyaluronan polymers, such as native hyaluronan, the esterified derivative of hyaluronan Hyaff 11p50 (where 50% of the carboxy groups of hyaluronic acid are esterified with benzyl alcohol) and the autocross-linked polymer (ACP) internally esterified derivative of hyaluronan, by solvent evaporation and spray-drying methods. As model drugs cromolyn sodium salt, metronidazole and prednisolone hemisuccinate sodium salt were employed. The influence of polymer and preparation procedure has been evaluated on microparticle characteristics (i.e. morphology and encapsulation yield) and on the drug release profiles. The use of solvent evaporation method, a polymeric matrix constituted of Hyaff 11p50 3% (w/v), a dispersing phase constituted of 80 g of mineral oil (w/o ratio: 0.1), Span 85 0.1% (w/w) as stabilizer, and a stirring speed of 700 rpm resulted in the production of microspheres characterized by spherical shape, absence of aggregates, a mean diameter of 6.4 μm and a recovery of 90% (w/w). The production of drug containing microspheres led to an increase of mean diameter of microspheres and to high encapsulation yields. Moreover in vitro models have demonstrated that in all cases drugs were released from Hyaff 11p50 microspheres in a controlled fashion. Finally mathematical analysis of the drug release modalities has evidenced that drug release from Hyaff 11p50 microspheres is more consistent with kinetics of the diffusion rather than of the dissolution type.

Keywords: Hyaff 11p50; Microspheres; Solvent evaporation; Spray-drying

1. Introduction

Hyaluronan (HA) is an abundant non-sulfated glycosaminoglycan component of synovial fluid and extracellular matrices. It can be considered as an attractive building block for new biocompatible and biodegradable polymers, having applications in drug delivery, tissue engineering, and viscosupplementation (Pouyani et al., 1994; Vercruysse and Prestwich, 1998). Nevertheless, the poor biomechanical properties of hyaluronan prevent the fabrication of new biomaterials. At this regard a variety of chemical modifications of native

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hyaluronan have been designed to provide mechanically and chemically robust materials (Abatangelo and Weigel, 2000; Kuo et al., 1991). The resulting hyaluronan derivatives have physicochemical properties significantly different from the native polymer, retaining the biocompatibility and biodegradability of original hyaluronan (Benedetti et al., 1993; Burns et al., 1996).

Esterified hyaluronan biomaterials have been prepared by alkylation of the tetra(*n*-butyl)ammonium salt of hyaluronan with an alkyl halide in dimethylformamide (DMF) solution (Luo et al., 2000). In particular Hyaff 11 at 50% of esterification is a hydrophilic polymer (water solubility 60 mg/ml), which may be processed as a gel, very similar to native hyaluronic acid, but with a higher resistance to the action of the hyaluronidases, the natural enzymes which degrade hyaluronic acid, forming shorter polysaccharidic chains (Pouyani et al., 1994). At higher percentages of esterification, the resulting Hyaff materials became insoluble in water (Larsen et al., 1991). These hyaluronan benzyl esters can be extruded to produce membranes and fibers, lyophilized to obtain sponges, or processed by spray-drying, extraction, and evaporation to produce microspheres (della Valle and Romeo, 1990; Callegaro et al., 1993; Lim et al., 2000; Elvassore et al., 2001).

Microspheres and thin films of hyaluronan benzyl esters have been found to be the most suitable physical forms for drug and peptide delivery, since the hydrophobic, mucoadhesive properties facilitated intranasal, buccal, ocular, and vaginal delivery (Bonucci et al., 1995; Richardson et al., 1995, 1996; Luo et al., 2000; Singh et al., 2001). In particular Richardson et al. have evaluated the vaginal absorption of salmon calcitonin in solution or in Hyaff 11 microspheres in rats. The authors have demonstrated that the hypocalcaemic responses were enhanced by the use of Hyaff 11 microspheres (Richardson et al., 1995). Moreover, a gamma-scintigraphy method has demonstrated the potential of Hyaff 11 microspheres as a long-acting intravaginal delivery system in sheep (Richardson et al., 1996).

Hyaluronan benzyl ester materials have been used as meshes and sponges for growth of cultured human fibroblasts and for culture of chondrocytes and bone-marrow derived mesenchymal cells for repair of cartilage and bone defects (Benedetti, 1994). The autocross-linked polymer (ACP) is an internally esterified derivative of hyaluronan, with both inter- and intra-molecular bonds between the hydroxyl and carboxyl groups of hyaluronan (Mensitieri et al., 1996). ACP can be lyophilized to a white powder and hydrated to a transparent gel (Prestwich et al., 1997). This novel biomaterial has been used as a barrier to reduce post-operative adhesions as a consequence of abdominal and gynecological surgery and as a scaffolding for cell growth repair of tissue defects (Yerushalmi et al., 1994). Cartilage and bone regeneration occurred in subcutaneously implantated porous sponges of ACP that had been seeded with chondrocytes or osteoblasts.

In this paper hyaluronans have been employed to produce microspheres, allowing the efficient encapsulation of different drugs, namely cromolyn sodium, metronidazole and prednisolone hemisuccinate sodium.

Chemically, cromolyn sodium is the disodium salt of 1,3-bis(2-carboxychromon-5-yloxy)-2-hydroxypropane. Cromolyn sodium is indicated as a component of therapy in the treatment of mild persistent and moderate persistent asthma by the Expert Panel II report (Murphy et al., 1997). Metronidazole, 1-(beta-hydroxyethyl)-2-methyl-5-nitroimidazole, is an oral synthetic antiprotozoal and antibacterial agent, it is indicated for trichomoniasis, anaerobic bacterial infections, bacterial vaginosis, Helicobacter pylori, antibiotic-associated diarrhea and colitis. Moreover topical administration of metronidazole may improve periodontal diseases (Van Dyke and Tohme, 2000; Al-Mubarak et al., 2000).

Prednisolone (17-ethylcarbonate 21-proprionate) is used to achieve prompt suppression of inflammation in many inflammatory conditions (e.g. rheumatoid arthritis, systemic lupus, acute gouty arthritis, psoriatic arthritis, ulcerative colitis, and Crohn's disease) and allergic conditions (e.g. bronchial asthma, allergic rhinitis, drug-induced dermatitis, contact and atopic dermatitis). Chronic allergic and inflammatory conditions of the uvea, iris, conjunctiva and optic nerves of the eyes are also treated with prednisolone (Esposito et al., 2000).

This report describes: (a) the production of biodegradable microparticles using different hyaluronan polymers such as native hyaluronan, the esterified hyaluronan biomaterials Hyaff 11p50 or ACP, (b) the influence of polymer and preparation procedure on microparticle characteristics (i.e. morphology and encapsulation yield) and finally (c) the effect of all these variables on the drug release profiles. Different drying

processes were utilized for the microparticle production namely based on solvent evaporation from a water-in-oil emulsion, or on spray-drying method. As model drugs cromolyn sodium salt, metronidazole and prednisolone hemisuccinate sodium salt were employed.

2. Materials and methods

2.1. Materials

Hyaluronic acid (HA), the esterified derivative of hyaluronan Hyaff 11p50 (where 50% of the carboxy groups of hyaluronic acid are esterified with benzyl alcohol) and the autocross-linked polymer internally esterified derivative of hyaluronan ACP were from Fidia Advanced Biopolymers (Padova, Italy). Cromolyn sodium salt (CROSS), Metronidazole (METR), Prednisolone hemisuccinate sodium salt (PRESS), Mineral oil and Span 85 were purchased from Fluka Chemie (Buchs, Switzerland).

2.2. Production of microparticles

Microparticles were alternatively produced by (a) solvent evaporation from a water-in-oil emulsion (w/o) or by (b) spray-drying methods.

2.2.1. Solvent evaporation from a water-in-oil emulsion

Typically, 300 mg (3% w/v) of polymer, alternatively represented by HA, Hyaff 11p50 or ACP were dissolved/suspended in 10 ml of bidistilled water. To this solution or suspension, 30 mg of CROSS, METR or PRESS were added. The obtained mixture was then dropwise emulsified with 80 g of mineral oil containing Span 85 (0.1%, w/w) as emulsifying agent. The emulsion was maintained at 40 °C under continuous stirring with an apparatus based on a 50 mm diameter vessel, a 35 mm and a four-blade turbine rotor (Eurostar digital IKA Labortechnik) at 700 rpm for 12 h. Afterwards the microspheres were separated from the oil phase by centrifugation at 2500 rpm for 15 min (Centrifuge Medifuge, Heraeus Sepatech). Microspheres were then washed several times with diethyl ether to remove excess oil, filtered and left to completely desiccate in Petri dishes. For the production of drug-loaded microparticles, microspheres were produced by the solvent evaporation method using as aqueous disperse phase Hyaff 11p50 3% (w/v), as dispersing phase mineral oil 80 g, a w/o ratio of 0.1 w/w, as stabilizer Span 85 0.1% (w/w) and a stirring speed of 700 rpm.

2.2.2. Spray-drying method

Microparticles were alternatively produced using a Buchi Mini Spray Dryer Model 190 (Buchi, Laboratoriums Technik AG, Flawil, Germany). Briefly, a polymer solution (150 or 300 mg in 10 ml of water) was fed into the instrument by a peristaltic pump and sprayed with a 0.7 mm nozzle, by means of a flow of compressed air, in the drying chamber of the apparatus. A flow of heated air aspirated by a pump induced the quick evaporation of the solvent from the drops, leading to the formation of solid microparticles.

In particular microspheres were obtained by nebulization of Hyaff 11p50 1.5% or 3% (w/v) and the following instrumental settings (a) feed rate of the polymer solution ("pump"), 5 ml/min (b) air flow rate of the nebulization device ("flow"), 600 l/h, (c) flow of drying air ("aspirator"), 28 m^3/h, (d) inlet air drying temperature ("heating") 115 °C, outlet temperature 86 °C.

The obtained particles, after separation from the exhausted air in a cyclone, settled into a bottom collector and were kept under vacuum. For the production of drug-loaded microparticles, 30 mg of cromolyn, metronidazole or prednisolone hemisuccinate were solubilized in the polymer solution before spray-drying.

2.3. Microparticle morphological analysis

Microparticle morphology was evaluated by electron microscopy. Dried microparticles were analyzed at 15–20 kV by a scanning electron microscope (360 Stereoscan Cambridge Instruments Ltd., Cambridge, UK) after metallization by gold coating (Edwards Sputter coating S 150). Size and size distributions were evaluated by optical microscopy, using an inverted microscope (Nikon Diaphot, Tokyo, Japan) equipped with a digital camera. Microspheres size and size distribution were determined considering the length diameter on digital photomicrographs. At least 1000 microspheres for each sample were measured, after suspending the sample in mineral oil plus 1% of Span 85 as dispersing agent. The count of micropheres was performed by choosing randomly six different fields. The observations were performed on five different batches of each

microparticle preparation. The analysis was performed by the computerised size analysis system "NIH Image", a public domain image processing and analysis program for the Macintosh.

2.4. Microparticle recovery

Microparticle recovery efficiencies were calculated as percentage of weight of the obtained microparticles, taking as reference the total amount of polymer used for the preparation. The percentage of recovery does not take into account the residual water and oil contents in the particles, these parameters have been disregarded because particles appear very dry and not greasy.

2.5. Drug content of microparticles

The amount of encapsulated drug per mg of dried microsphere was determined by evaluating the unentrapped drug concentration. In particular 250 ml of diethyl ether were added to the surnatant obtained after microsphere centrifugation (mineral oil) combined with the washing solution (diethyl ether). The resulting organic layer was extracted twice with 30 ml of distilled water. The concentration of drug in the aqueous phase or in the organic phase was then determined by UV spectroscopy (Perkin-Elmer Lambda-19 Spectrophotometer). The considered wavelength values correspond to the λ_{max} of the different drugs, namely 326, 319 and 242 nm for CROSS, METR and PRESS, respectively.

2.6. In vitro release kinetics

The in vitro release tests were carried out with two different experimental approaches under different conditions.

2.6.1. Horizontal shaker method

The test was carried out as previously described (Nastruzzi et al., 1993). Typically, 30 mg of microspheres were placed into a dialysis tube (molecular weight cut off 10,000–12,000; Medi Cell International, UK), then placed into 30 ml of isotonic borate buffer (IPB) (H_3BO_3 180 mM, $Na_2B_4O_7{\cdot}10H_2O$ 5 mM, NaCl 18 mM, pH 7.4) and shaken in a horizontal shaker. Throughout, samples were withdrawn at regular time intervals from the external buffer and the amount of released drug was determined by UV spectroscopy.

2.6.2. Flow-through cell method

Alternatively, a column elution method was used to measure in vitro drug release. About 60 mg of dry microspheres were placed into a plexy glass column filled with 6 ml of IPB. At the bottom of the column was placed a polyethylene filter to prevent microspheres leakage and both ends were fitted with Teflon tubing. Care was taken to ensure the quantitative transferring of microspheres to the column. IPB was pumped through the column at a flow rate of 1 ml/min by a peristaltic pump (Miniplus 2 GILSON, Abimed, UK). Fractions were collected and analyzed for drug content by UV spectroscopy after dilution with borate buffer.

2.7. Drug release data analysis

The experimental release data obtained with both methods were then fitted to the following semiempirical equations respectively describing Fickian dissolutive and diffusional release mechanisms from microspheres (Nastruzzi et al., 1993):

$$M_t/M_\infty = K_{\mathrm{Diss}} t^{0.5} + c \quad (1)$$

$$\left(1 - \frac{M_t}{M_\infty}\right) = \mathrm{e}^{-K_{\mathrm{Diff}} t} + c \quad (2)$$

where M_t/M_∞ represents the drug fraction released at the time t, (M_∞ is the total drug content of the analyzed amount of microspheres), K and c are coefficients calculated by plotting the linear forms of the indicated equations. The release data up to the plateau of percent of released drug were used to produce theoretical release curves.

3. Results and discussion

3.1. Production of microspheres by solvent evaporation

As first approach, hyaluronan microspheres were prepared by a solvent evaporation technique. The choice and the adjustment of the manufacturing parameters for the production of microspheres of defined size were performed in agreement with the following

equation:

$$d \propto K \frac{D_v R \upsilon_a \gamma}{D_s N \upsilon_o C_s} \quad (3)$$

where d is the average particle size, K a variable depending on the apparatus geometry (e.g. type and dimension of stirrer), D_v and D_s are respectively the diameter of the vessel and of the stirrer, R the volume ratio between aqueous and oil phases, υ_a and υ_o their respective viscosities, N the stirring speed, γ the surface tension between the two immiscible phases and C_s the stabilizer concentration (Arshady, 1990).

The influence of some parameters such as polymer type and concentration, oil phase volume, surfactant concentration and stirring speed was studied on morphology, mean diameter, dimensional distribution and recovery efficiency of microparticles.

3.1.1. Polymer type and concentration

Microparticles were firstly produced by the use of HA. During the emulsification of the polymer solution in the oily phase (alternatively constituted of mineral oil or isopropyl palmitate) optical microscopy observation revealed the presence of microparticles, nevertheless no isolating strategy based on the use of different washing solvents (e.g. hexan, ethanol and ethyl acetate) could result in the obtainment of a dry microparticle powder. The formation and stabilization of microsphere are related to chemistry, thus the failure to formulate microspheres using native HA can be attributed to its chemistry rather than to its mechanical properties (Prestwich et al., 1997).

As second approach the autocross-linked polymer ACP (0.5% w/v) was employed. The aqueous dispersion of ACP, being highly viscous, prevented to obtain microparticles, leading to an inter-particle adhesion during the emulsification step and in a final aggregated semisolid system.

Microparticles were then produced employing aqueous solutions of Hyaff 11p50, in concentrations comprised between 1.5 and 5% (w/v). The use of this polymer allowed to obtain microparticles easily to isolate by centrifugation followed by washing with diethyl ether. The final dry product appeared as a flowing powder.

In particular the use of Hyaff 11p50 1.5% (w/v) resulted in spherical microspheres with a slightly irregular surface, a mean diameter of 3 μm and a recovery of 66.6% (w/w) with respect to the weight of polymer utilized. A double polymer concentration led to spherical microspheres with a smooth surface, a double length microsphere diameter (6.4 μm) and an almost quantitative recovery (90%, w/w). Finally the highest polymer concentration (5% w/v) prevent microspheres isolation, only resulting in several polymer aggregates after centrifugation. Hyaff solutions with higher concentration (6% w/v) are characterized by a viscosity too high to be employed as disperse phase in the solvent evaporation method. Table 1 summarizes the effect of Hyaff 11p50 concentration on some microsphere characteristics. Fig. 1 shows some scanning electron micrographs and frequency distribution plots of microspheres produced by different Hyaff 11p50 concentration.

3.1.2. Oil phase volume

As external dispersing phase different volumes of mineral oil (50, 80 or 100 g) were employed, resulting in different ratios between aqueous internal and oil external phases (w/o ratio), namely 0.16, 0.1 and 0.08. Table 2 summarizes for comparison the obtained results. Hyaff 11p50 concentration was 3% (w/w). The use of the lower amount of oil (50 g) led to formation of irregularly collapsed microparticles (Fig. 2A) with a mean diameter of 8.53 μm and a recovery efficiency of 77% (w/w). The highest amount of oil (100 g) led to a collapse of particles after isolation. Conversely particles produced by a 0.1 w/o ratio (80 g mineral oil) enabled the production of spherical microparticles, as shown in Fig. 2B, a mean diameter of 6.4 μm and a recovery efficiency of 90% (w/w). The decrease of w/o ratio resulted in an increase of particles diameter, in agreement with the Arshady Eq. [3]. The increase of particle diameter could be related to a concurrent increase of the droplet size of the precursory w/o emulsion before particle formation.

3.1.3. Surfactant type and concentration

The effect of the concentration of Span 85 was studied on size, morphology and recovery of microspheres produced by 3% (p/v) Hyaff 11p50 and 0.1 w/o ratio.

The addition of surfactants led to a reduction of the size of the Hyaff 11p50 droplets during the emulsification step, causing a significant decrease of the final microsphere size (Table 3). It was found that the higher the concentration of Span 85, the smaller the mean diameter of the obtained microspheres, passing from 15 μm

Table 1
Effect of polymer concentration on microsphere characteristics

Hyaff (%)	w/o ratio	Mean diameter[a] (μm) ± S.D.	Mean recovery[a,b] (%) ± S.D.	Notes
1.5	0.10	3.04 ± 0.8	66.6 ± 0.7	Spherical shape
3	0.10	6.40 ± 0.9	90.0 ± 1	Spherical shape
5	0.10	n.d.	n.d.	Several aggregates

n.d.: Not determined. Microspheres were produced by the solvent evaporation method using as dispersing phase: mineral oil 80 g, as stabilizer: Span 85 0.1% (w/w) and stirring speed: 700 rpm.

[a] Data represent the mean of six independent experiments.

[b] Percentage of weight of microparticle recovered with respect to weight of polymer utilized.

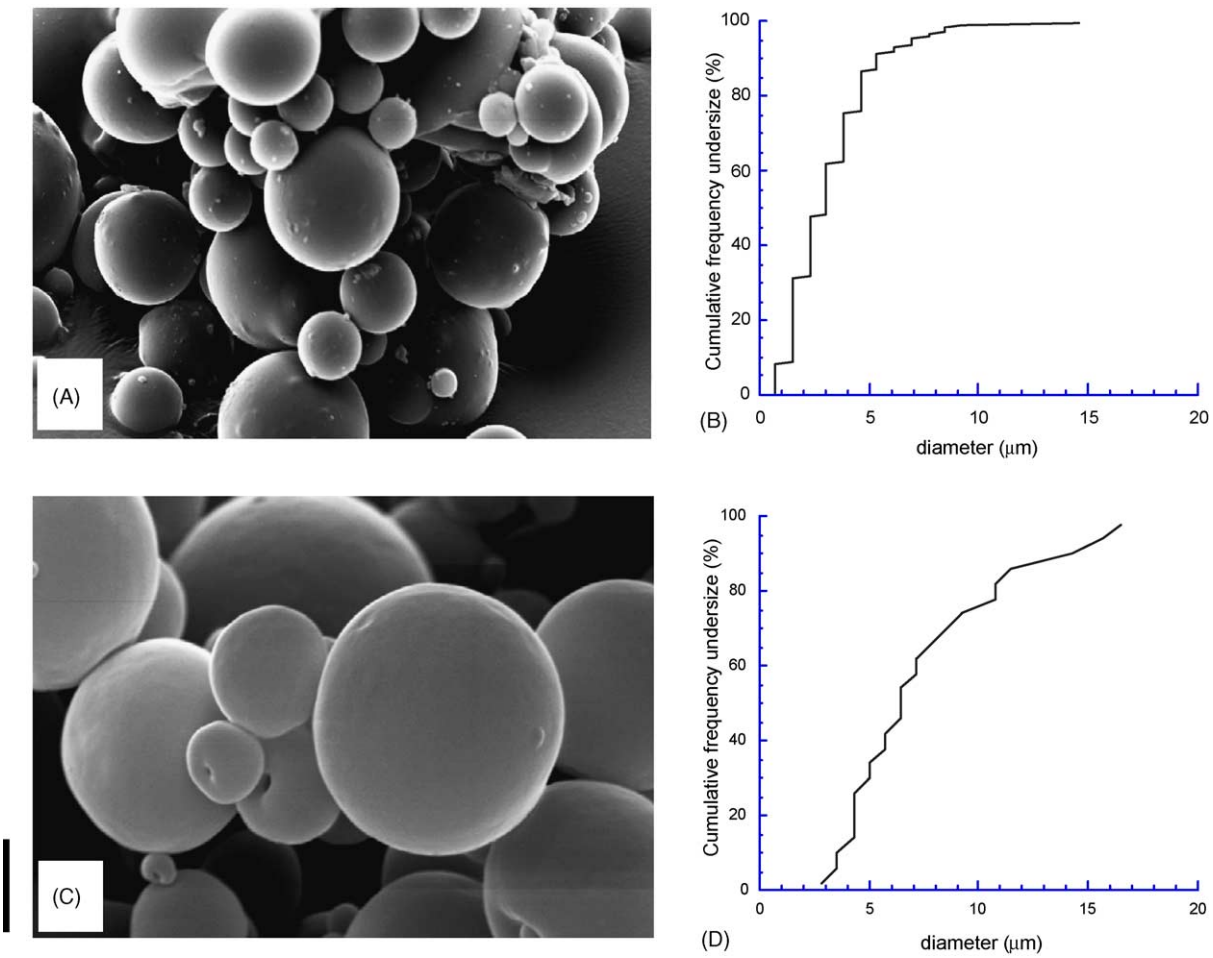

Fig. 1. Scanning electron micrographs (A, C) and dimensional frequency distribution plots (B, D) of microparticles constituted of Hyaff 11p50 1.5% (w/w) (A, B), or 3% (w/w) (C, D). The bar equals 2.3 and 2.9 μm in panels A and C, respectively. Microparticles were obtained by the solvent evaporation method and the standard conditions defined in the text.

Table 2
Effect of the amount of oil phase on microsphere characteristics

Mineral oil (g)	w/o ratio	Mean diameter[a] (μm) ± S.D.	Mean recovery[a,b] (%) ± S.D.	Notes
50	0.16	8.53 ± 0.4	77 ± 0.8	Irregular shape
80	0.10	6.40 ± 0.8	90 ± 0.5	Spherical shape
100	0.08	n.d.	n.d.	Collapsed particles

n.d.: Not determined. Microspheres were produced by the solvent evaporation method using an aqueous disperse phase of Hyaff 11p50 3% (w/v) as stabilizer: Span 85 0.1% (w/w) and stirring speed: 700 rpm.

[a] Data represent the mean of six independent experiments.

[b] Percentage of weight of microparticle recovered with respect to weight of polymer utilized.

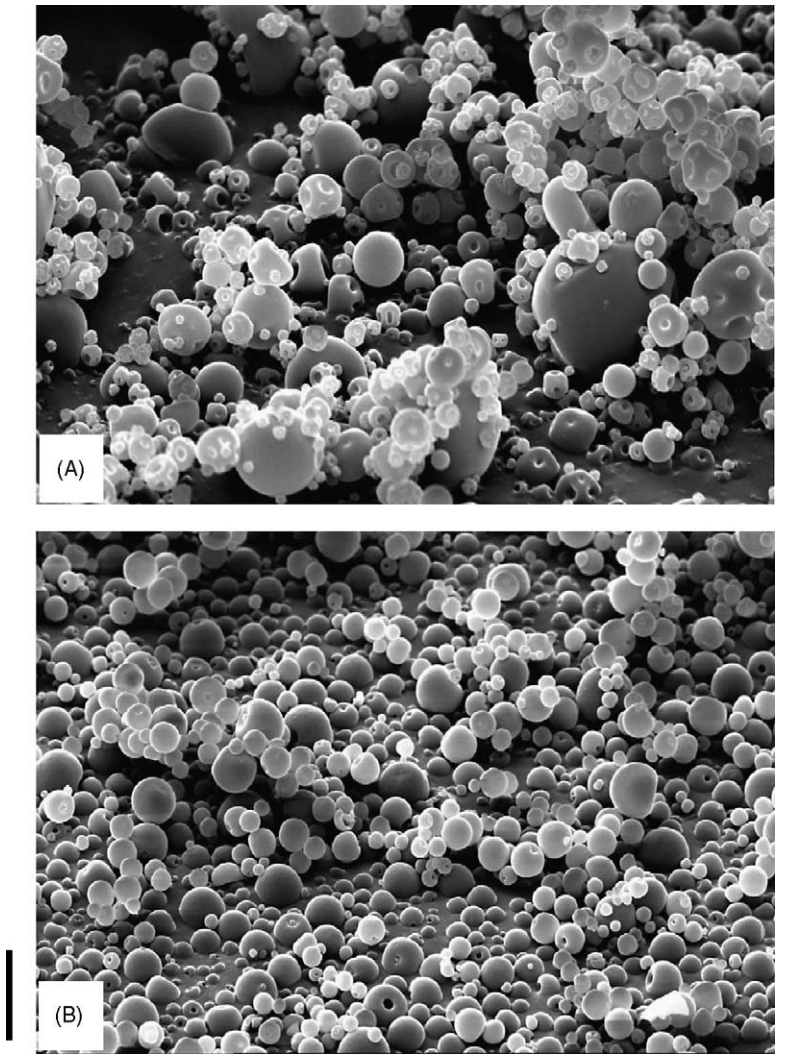

Fig. 2. Scanning electron micrographs of Hyaff 11p50 microparticles obtained by the solvent evaporation method using a w/o ratio of 0.16 (50 g of mineral oil) (A) or 0.1 (80 g of mineral oil) (B). Microparticles were obtained by the solvent evaporation method and the standard conditions defined in the text. The bar equals 14.21 and 16 μm in panels A and B, respectively.

Table 3
Effect of the stabilizer concentration on microsphere characteristics

Span 85 (% w/w)	w/o ratio	Mean diameter[a] (μm) ± S.D.	Mean recovery[a,b] (%) ± S.D.	Notes
0.05	0.10	15.00 ± 1	43 ± 0.9	Irregular shape
0.1	0.10	6.40 ± 0.8	90 ± 0.5	Spherical shape
0.2	0.10	5.00 ± 0.4	70 ± 0.6	Spherical shape
0.4	0.10	2.60 ± 0.5	80 ± 0.3	Regular shape

Microspheres were produced by the solvent evaporation method using as aqueous disperse phase Hyaff 11p50 3% (w/v), as dispersing phase mineral oil 80 g and as stirring speed 700 rpm.
[a] Data represent the mean of six independent experiments.
[b] Percentage of weight of microparticle recovered with respect to weight of polymer utilized.

in the case of 0.05% (w/w) of stabilizer to 2.6 μm in the case of 0.40% (w/w).

The best results in term of microsphere shape were obtained in the case of Span 85 0.1 and 0.2% (w/w).

The use of the lowest stabilizer concentration (0.05%, w/w) led to the formation of large droplets in the water-in-oil emulsion tending to aggregate together. This phenomenon resulted in the lowest microparticle recovery (43%, w/w). Taken together these results confirm the observations reported in previous papers by Esposito et al. (1996, 2001).

3.1.4. Stirring speed

Variation of stirring speed had a strong influence on Hyaff 11p50 microparticle production. In fact using 3% (p/v) Hyaff 11p50, 0.1 w/o ratio and 0.1% (w/w) Span 85, it was found that a 500 rpm stirring speed was too low to obtain structured microspheres. During solvent evaporation microspheres appear very big at the optical microscope (tentatively diameter 300–400 μm) only resulting in collapsed beads after isolation. On the contrary, a double stirring speed, namely 1000 rpm, led to the production of spherical microspheres, characterized by 3.3 μm mean diameter and 77% (w/w) recovery (Table 4). These findings are in agreement with results published by Arshady (1990) and Esposito et al. (1996). Nevertheless the vorticose motion caused by the high stirring speed led to a loss of polymer droplets out from the beaker during microsphere production, finally resulting in a decrease of recovery.

The best results in term of recovery were obtained by the use of 700 rpm stirring speed (90%, w/w), microspheres in this condition were spherical, with a 6.4 μm mean diameter.

At last the "standard conditions" for microsphere production by solvent evaporation have been assessed: (a) a polymeric matrix constituted of Hyaff 11p50 3% (w/v), (b) a dispersing phase constituted of 80 g of mineral oil (w/o ratio: 0.1), (c) Span 85 0.1% (w/w) as stabilizer, and finally (d) a stirring speed of 700 rpm. In these conditions the obtained microspheres were characterized by spherical shape, absence of aggregates, a mean diameter of 6.4 μm and a recovery of 90% (w/w).

3.2. Production of microspheres by spray-drying

As second approach microspheres have been produced by the spray-drying method (Broadhead et al., 1992; Esposito et al., 2000). Fig. 3 shows the morphology and the dimensional distribution of microparticles

Table 4
Effect of the stirring speed on microsphere characteristics

Stirring speed (rpm)	w/o ratio	Mean diameter[a] (μm) ± S.D.	Mean recovery[a,b] (%) ± S.D.	Notes
500	0.10	n.d.	n.d.	Collapsed particles
700	0.10	6.40 ± 0.8	90 ± 0.5	Spherical shape
1000	0.10	3.33 ± 0.9	77 ± 1	Spherical shape

n.d.: Not determined. Microspheres were produced by the solvent evaporation method using as aqueous disperse phase Hyaff 11p50 3% (p/v), as dispersing phase mineral oil 80 g, and as stabilizer Span 85 0.1% (w/w).
[a] Data represent the mean of six independent experiments.
[b] Percentage of weight of microparticle recovered with respect to weight of polymer utilized.

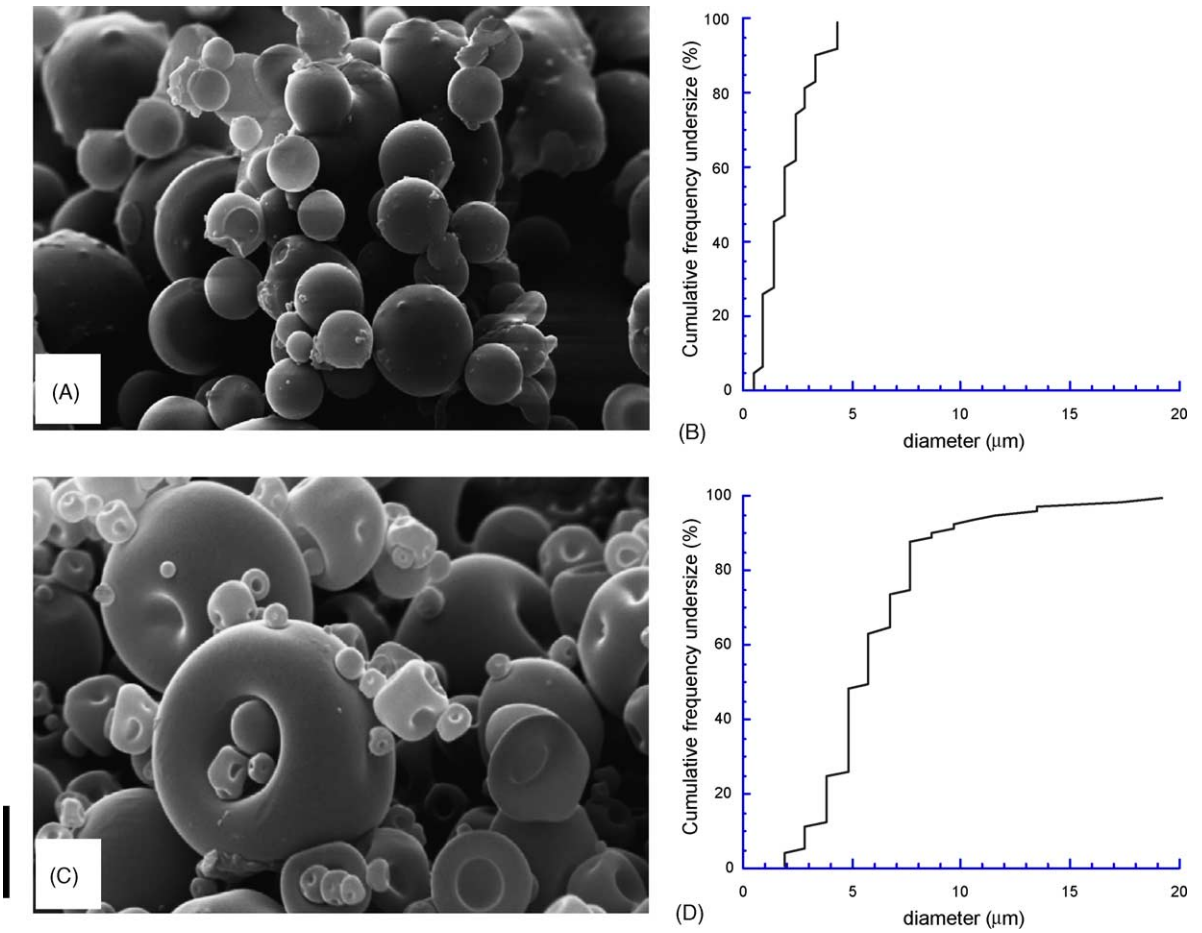

Fig. 3. Scanning electron micrographs (A, C) and dimensional frequency distribution plots (B, D) of microparticles constituted of Hyaff 11p50 1.5% (w/w) (A, B), or 3% (w/w) (C, D). The bar equals 1.65 and 5.27 μm in panels A and C, respectively. Microparticles were obtained by the spray-drying method.

produced by spray-drying and different Hyaff 11p50 concentrations, Table 5 summarizes some characteristics of the obtained particles. The use of the lower polymer concentration led to spherical particles with a smooth surface (Fig. 3A) a mean diameter of 1.88 μm and a narrow dimensional distribution, on the other hand the higher concentration resulted in formation of microspheres presenting a hole, the so-called "cenospheres" (Sacchetti and Van Oort, 1996; Cortesi et al., 2003) (Fig. 3B), a larger mean diameter (5.27 μm) and a broader dimensional distribution. Concerning recovery efficiency, both Hyaff 11p50 concentrations resulted in low values, as reported in Table 5. This drawback can be attributed to the high bioadhesivity of the polymer

Table 5
Effect of the polymer concentration on the characteristics of microspheres produced by spray-drying method

Hyaff 11p50 (%)	Mean diameter[a] (μm) ± S.D.	Mean recovery[a,b] (%) ± S.D.	Notes
1.5	1.88 ± 0.3	13 ± 0.8	Spherical shape
3	5.27 ± 0.9	23 ± 0.5	Cenosphere

[a] Data represent the mean of six independent experiments.
[b] Percentage of weight of microparticle recovered with respect to weight of polymer utilized.

that tends to adhere to the desiccator camera, minimizing the microsphere recovery.

In the light of the above reported results solvent evaporation was considered the best method for Hyaff 11p50 microsphere production.

3.3. Drug encapsulation

By the end of the preformulation study, Hyaff 11p50 microspheres have been produced by solvent evaporation and the "standard conditions" in the presence of different drugs. In particular cromolyn sodium salt (CROSS), metronidazole (METR) and prednisolone hemisuccinate sodium salt (PRESS) have been encapsulated in Hyaff 11p50 microspheres produced by the solvent evaporation method and the standard conditions previously assessed.

Fig. 4 shows the morphology of the drug containing microspheres together with their dimensional distribution, Table 6 reports some characteristics of the microspheres.

In all cases microspheres are characterized by a spherical shape. Concerning surface, microspheres exhibit an irregular surface in the presence of CROSS and a smooth one in the presence of METR, while PRESS containing microspheres show some spherical prominences on the surface.

Mean diameter of Hyaff 11p50 microspheres has been significantly affected by the presence of drugs, especially in the case of CROSS and METR, where diameters were respectively 5 and 2.4 times higher than empty microspheres. The presence of PRESS caused a minor increase of mean diameter of microspheres, passing from 6.4 to 10 μm.

As previously found in other studies (Esposito et al., 2001; Cortesi et al., 2002), one can observe that the presence of drug generally results in an increase of particle size, this behaviour can be possibly related to a corresponding increase in the precursor w/o emulsion droplet size.

Particles were considered to be of a suitable size for nasal administration by insufflation (Lim et al., 2000; Chien et al., 1989) or for peroral administration into the periodontal pocket (e.g. METR containing microspheres) (Esposito et al., 1997).

Drug encapsulation yield was calculated by evaluating the unentrapped drug concentration. To this aim the amount of drug present in the surnatant and in the washing solutions obtained after microspheres isolation has been extracted by water and diethyl ether. In particular samples of aqueous phase have been analyzed by UV spectroscopy for the presence of CROSS and PRESS while organic phase was analyzed for the presence of METR.

With respect to the encapsulation efficiency exploited by Hyaff 11p50 microspheres, it should be stressed the crucial role of the chemico-physical characteristics of the utilized drug. In particular, the hydrophobic–hydrophilic balance of the drug molecule was found to influence the entrapment yield. Indeed, hydrophilic drugs can be quantitatively incorporated in microspheres, whilst molecules with hydrophobic portions display a reduced trapping efficiency. For instance, CROSS, being highly hydrophilic and almost insoluble in all organic solvents, displayed a very high encapsulation efficiency (98%), as much as PRESS (97%) whereas METR, having greater lipophilic portions, showed lower entrapment yields (85%) (Table 6). This behaviour can be attributed to a partial diffusion of drug molecules during the emulsification step, from the aqueous droplets to the external continuous oil phase.

Table 6
Characteristics of drug containing Hyaff microspheres

Drug[a]	Mean diameter[b] (μm)	Mean recovery[b,c] (%) ± S.D.	Mean encapsulation yield[b,d] (%) ± S.D.	Notes
CROSS	23.3 ± 0.1	92 ± 0.4	98 ± 0.5	Spherical shape irregular surface
METR	15.3 ± 0.8	89 ± 0.3	85 ± 0.4	Spherical shape smooth surface
PRESS	10.0 ± 0.6	90 ± 0.5	97 ± 0.7	Spherical shape prominences on the surface

[a] CROSS: cromolyn sodium salt; METR: metronidazole; PRESS: prednisolone hemisuccinate sodium salt.
[b] Data represent the mean of six independent experiments.
[c] Percentage of weight of microparticles recovered with respect to weight of polymer utilized.
[d] Percentage of encapsulated drug with respect to the total amount used.

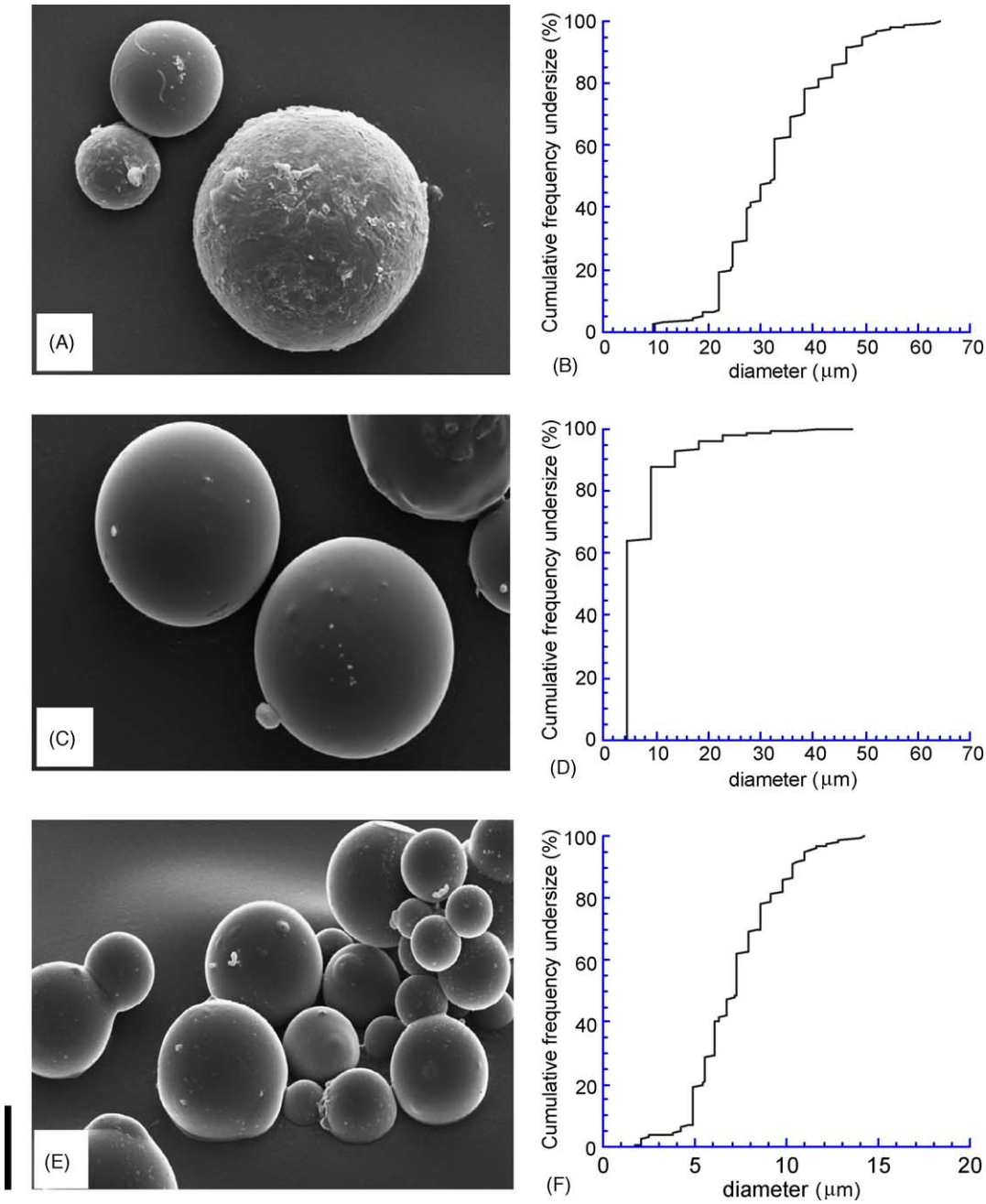

Fig. 4. Scanning electron micrographs (A, C, E) and dimensional frequency distribution plots (B, D, F) of Hyaff 11p50 microparticles containing CROSS (A, B), METR (C, D) or PRESS (E, F). The bar equals 16.15, 6.75 and 8.33 μm in panels A, C and E, respectively. Microparticles were obtained by the solvent evaporation method and the standard conditions defined in the text.

3.4. In vitro release of drug from Hyaff 11p50 microspheres

In vitro release profiles give important informations on the efficiency of the delivery system for the controlled release of drugs. An "in vitro" drug release study is indeed a prerequisite to obtain correct predictions in order to design and test the "in vivo" activity of controlled drug delivery forms (Nastruzzi et al., 1993).

In literature drug release kinetics from microparticles are often determined by a single in vitro experimental approach. In the present investigation two experimental approaches were employed, to determine if their alternative use could lead to different results. Flow through cell and horizontal shaker methods were employed to study drug release pattern from Hyaff 11p50 particles.

Usually, in vitro drug release and mathematical analysis are carried out under sink conditions. Nevertheless, in vitro systems should be predictive of in vivo "real" situation, where sink conditions are not always predominant. This consideration is especially important for the design of in vivo tests evaluating the effectiveness of the drug carrier system (Nastruzzi et al., 1993).

The two systems used in this study are alternatively predictive of sink and non-sink conditions. The flow through cell method on one hand mimics sink conditions, being a dynamic system where particles are continuously in contact with fresh elution medium. On the other hand, the horizontal shaker method reproduces a situation where non-sink conditions are predominant (Washington, 1990). In particular in the present study, particles are suspended in a relatively small volume of receiving medium (30 ml) in order to reproduce topical administration of Hyaff 11p50 microspheres (e.g. the nasal, the periodontal or the vaginal ones).

Fig. 5 reports release kinetics of CROSS, METR and PRESS from Hyaff microparticles, alternatively determined by dialysis or flow through cell method. The amount of drug released (expressed as a percentage of the amount of encapsulated drug) is plotted versus time. Data represent the mean of six independent experiments. Analyzing the release profiles, one can observe that by the first approach the plateau is reached approximately after 7 h, whilst by the second the plateau is reached more rapidly, namely after 25–30 min. Different drugs display different release curve shape, due to their chemico-physical characteristics. In particular in the case of CROSS, 40% of release is reached in about 6 h with the dialysis cell method and in 8.5 min with the flow through cell one (Fig. 5, panels A and B). In the case of METR, 40% of release is reached in 1 h or in 7 min, respectively (Fig. 5, panels C and D). Finally 40% of PRESS is released in 2.5 h by the first method or in 5 min by the second (Fig. 5, panels E and F).

It is to be noted that in all cases drugs were released from Hyaff 11p50 microspheres in a controlled fashion, even if the flow through cell method led to a more rapid drug release. Defining as "burst release" a release of 30% of drug within the first two time points, in the case of CROSS and PRESS there is an almost absence of the initial burst release phase, whilst in the case of METR the release is more rapid. These results differ from those obtained by Lim et al. (2000) concerning HA microsphere possibly because of the different nature of the polymer. Hyaff 11p50 infact, being a hyaluronan benzyl ester, does not dissolve rapidly in water as the native HA does, but is able to form a gellified network from which drug release can be controlled.

The use of two different methods to study drug release kinetics has indicated that parenteral administration (i.e. intramuscular or subcutaneous) of Hyaff 11p50 microspheres, reproduced by flow through cell method, would result in a rapid drug release, due to the water solubility of the polymer. Indeed Hyaff 11p50 microspheres, due to the high mucoadhesivity of the polymer, could be proposed for topic mucosal administration (Bonucci et al., 1995; Singh et al., 2001).

3.5. Mathematical analysis of the drug release modalities

The theoretical release curves were determined according to the linear form of Eq. (1), mimicking a dissolutive model and Eq. (2), mimicking a diffusive model (Peppas, 1985). Table 7 reports the parameters (K, c and R) determined by linearization of release rate data.

From the reported values it is therefore evident that both the CROSS, METR and PRESS release from Hyaff 11p50 microspheres appear to be more consistent with kinetics of the diffusion rather than of the dissolution type, either in the case of flow through cell or dialysis method, on the bases of the higher value of R found in the case of linearization of Eq. (2). This behaviour suggests a similarity to release from a matrix.

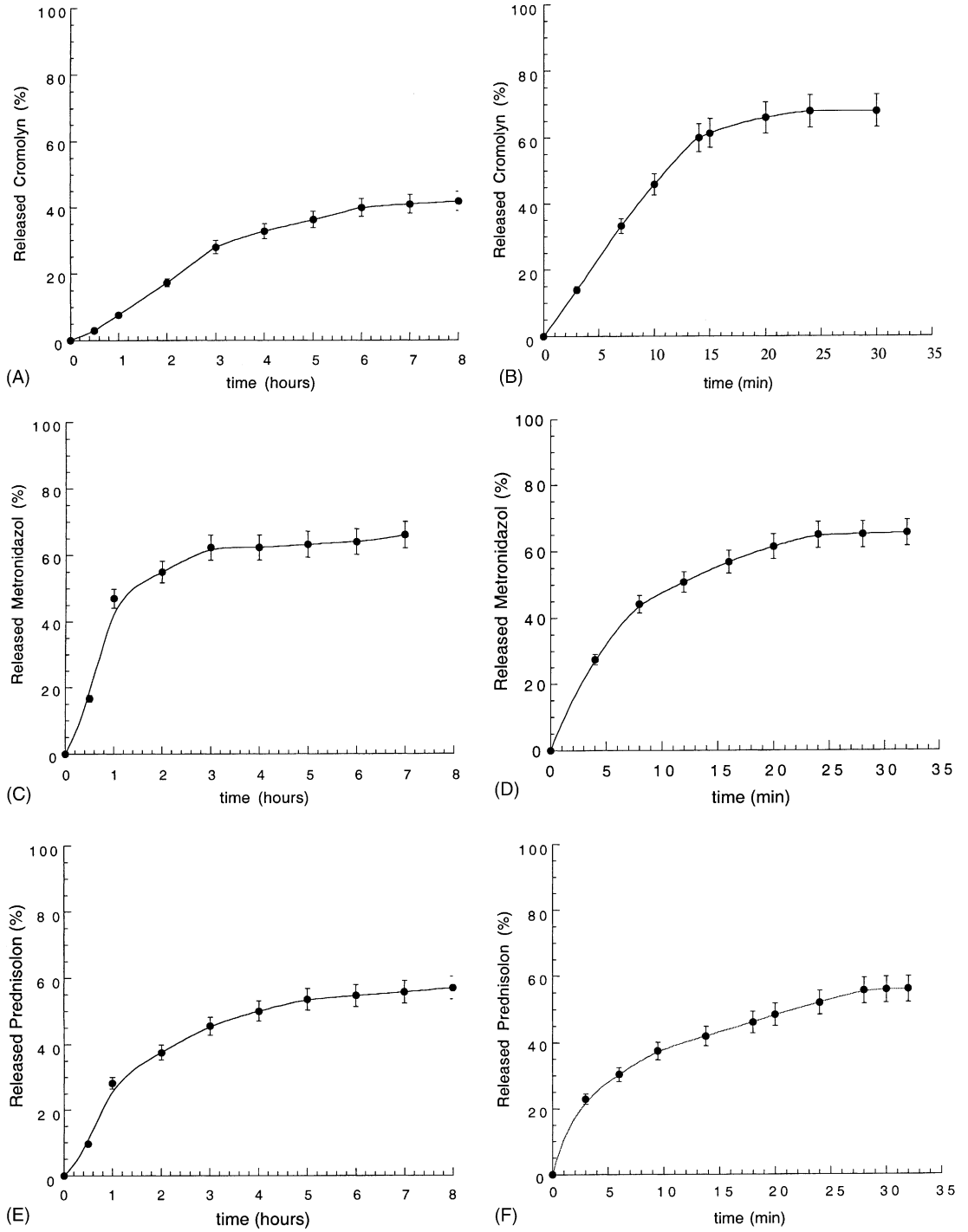

Fig. 5. Release profiles of CROSS (A, B), METR (C, D) or PRESS (E, F) from Hyaff 11p50 microparticles obtained by the solvent evaporation method and the standard conditions defined in the text. Kinetics were determined by dialysis (A, C, E) or flow through cell (B, D, F) methods. Data represent the average of six independent experiments.

Table 7
Release kinetic parameters of drug release from Hyaff 11p50 microparticles

Equation	Drugs[a]		
	CROSS	METR	PRESS
Dialysis method			
$(1-\frac{M_t}{M_\infty}) = e^{-K_{Diss}t} + c$			
K	−0.073	−0.109	−0.101
c	4.566	4.227	4.432
R	0.948	0.800	0.926
$M_t/M_\infty = K_{Diff}t^{0.5} + c'$			
K	17.743	22.376	21.504
c	−5.426	12.272	2.561
R	0.964	0.889	0.969
Flow through cell method			
$\left(1-\frac{M_t}{M_\infty}\right) = e^{-K_{Diss}t} + c$			
K	−0.037	−0.033	−0.017
c	4.493	4.418	4.404
R	0.958	0.933	0.795
$M_t/M_\infty = K_{Diff}t^{0.5} + c'$			
K	12.888	12.139	8.323
c	0.569	4.254	6.441
R	0.985	0.980	0.924

[a] CROSS: cromolyn sodium salt; METR: metronidazole; PRESS: prednisolone hemisuccinate sodium salt.

When hydrophilic polymer based microspheres such as Hyaff 11p50 are immersed in an aqueous medium, they swell and form a gel diffusion layer that hampers the outward transport of the drug within the matrix, hence producing a controlled release effect (Lim et al., 2000).

4. Conclusions

A preformulation study aimed to produce Hyaff 11p50 microspheres by a solvent evaporation method has been presented. Appropriate experimental conditions result in the production of Hyaff 11p50 based microspheres characterized by spherical shape, absence of aggregates, a mean diameter of 6.4 μm and an almost quantitative recovery.

Rochira et al. (1996) have performed a study on the production of microspheres based on the hyaluronan benzyl ester Hyaff 11, where HA is totally esterified with benzyl alcohol. Due to the total insolubility in water of Hyaff 11, microspheres have been produced by the use of a solvent extraction method and organic solvents such as dimethylsulphoxide, ethyl acetate and n-hexane. That method required different steps to remove residual solvents. Conversely Hyaff 11p50, due to its water solubility needs a simple protocol to produce microspheres characterized by morphology and dimensions similar to Hyaff 11 microspheres.

The encapsulation of drugs in Hyaff 11p50 microspheres caused an increase in mean diameter, especially in the case of CROSS and METR. Nevertheless microspheres had appropriate size for nasal administration by insufflation (Lim et al., 2000; Chien et al., 1989), for peroral administration into the periodontal pocket (Esposito et al., 1997) or for vaginal application (Bonucci et al., 1995). The use of different in vitro models have demonstrated that in all cases drugs can be released from Hyaff 11p50 microspheres in a controlled fashion. Hyaff 11p50 in fact does not dissolve in water as rapidly as the native HA, but it is able to form a gellified network from which drug can slowly diffuse.

Bioactivity experiments to investigate in vivo the performances of drug containing Hyaff 11p50 microspheres are now in progress.

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