International Journal of Pharmaceutics 367 (2009) 127–132

Contents lists available at ScienceDirect

International Journal of Pharmaceutics

journal homepage: www.elsevier.com/locate/ijpharm

Alginate/chitosan microparticles for tamoxifen delivery to the lymphatic system

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ARTICLE INFO

Article history:
Received 4 July 2008
Received in revised form
17 September 2008
Accepted 22 September 2008
Available online 1 October 2008

Keywords:
Lymphatic system
Microparticles
Tamoxifen
Alginate
Chitosan

ABSTRACT

Oral administration of the nonsteroidal anti-estrogen tamoxifen (TMX) is the treatment of choice for metastatic estrogen receptor-positive breast cancer. With the aim to improve TMX oral bioavailability and decrease its side effects, crosslinked alginate microparticles for the targeting to the lymphatic system by Peyer's patch (PP) uptake were developed and *in vitro* characterized. TMX was molecularly dispersed inside the microparticles and an electrostatic interaction involving the TMX tertiary amine was detected by rheological and FT-IR assays. Microparticles showed a size less than 3 μm, then suitability to be taken up by M cells in PP and a positive surface charge. Moreover, TMX loading level as well as *in vitro* release behaviour was affected by the polymer network connected with the mannuronic/guluronic ratio of the alginate chains.

1. Introduction

Oral administration of the nonsteroidal anti-estrogen tamoxifen citrate salt (TMX) is the treatment of choice for metastatic estrogen receptor-positive breast cancer. It is employed for the long-term prophylactic therapy in high-risk and post-menopausal women as well as for the treatment of advanced or metastatic breast cancer (Buckley and Goa, 1989; 11th Report on Carcinogens, 2005). TMX is administered as tablets or oral solutions at the dose of 10 mg twice a day or 20 mg once a day. However, following long-term therapy, TMX increased the incidence of vaginal symptoms, thromboembolic events, stroke and endometrial cancer (Shin et al., 2006) and the development of drug resistance (Maillard et al., 2005). Moreover, TMX showed a fairly good oral biovailability combined with large interindividual variations and extensively liver metabolization leading to increased dose and, consequently, side effects (McVie et al., 1986; Tukker et al., 1986).

Therefore, TMX formulation in micro or nanoparticles aiming to increase the oral biovailability or to achieve the desired dose at tumour site for a longer period were studied. Polyethylene glycol-coated nanospheres were proposed, resulting in an immediate drug release (Brigger et al., 2001). Poly(ε-caprolactone) nanoparticles were rapidly internalized in MCF-7 cells and they increased the local concentration of TMX in estrogen receptor-positive breast cancer cells (Chawla and Amiji, 2003). More, a formulation of microspheres of poly lactide-co-glycolide was designed to sustain the TMX action (Shera and Dhake, 2005) and the drug dosing by hydroxybutenyl-β-cyclodextrin provided a very significant increase in TMX oral bioavailability (Buchanan et al., 2006).

The targeting to the gut-associated lymphoid tissue (GALT), through M cells in Peyer's patches (PP), by a microparticulate carrier represents another possible strategy to overcome TMX limitations. Peyer's patch M cells, which are specialized cells staying over mucosa-associated lymphoid tissue (MALT), interspersed by enterocytes in the follicle-associated epithelium (FAE), can transport via endocytosis a variety of microparticulate matter from the gut lumen to intra-epithelial lymphoid cells and subsequently through the lymphatic system into the blood stream (Florence, 1997; Clark et al., 2001; Hussain et al., 2001). Therefore, TMX delivery to the GALT by the oral administration of microparticles could increase the drug bioavailability, avoid the enzymatic degradation from enterocytes as well as the first-pass metabolism so decreasing the dose and, consequently, the toxic effects. Furthermore, since the lymph system is one of the ways in which breast cancer can spread (stage 2), the targeting to the GALT could represent an advantageous approach to prevent the stage 2 of the illness.

Calcium alginate/chitosan (CaA/CHT) microparticles were previously designed exhibiting resistance to gastro-intestinal media, mucoadhesiveness and ability to be taken up by PP, even if the involvement of transport pathways across villous enterocytes was noticed (Coppi et al., 2006).

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doi:10.1016/j.ijpharm.2008.09.040

Thus, calcium alginate/chitosan microparticles loaded with TMX were prepared by crosslinking processes with calcium ions and chitosan carried out on alginate microparticles obtained by spray-drying sodium alginate/TMX water solutions. To evaluate the suitability of the delivery system for the TMX GALT targeting, the crosslinked microparticles were *in vitro* characterized for shape, size, charge surface, IR and DSC patterns, alginate/TMX interaction, TMX loading and release. Moreover, the influence of alginate mannuronic/guluronic ratio, affecting a possible interaction with cationic drugs, on the above properties was examined by using two types of sodium alginate in the microparticle preparation.

2. Materials and methods

2.1. Materials

The following chemicals were obtained from commercial suppliers and used without further purification. Sodium alginate (Relative Molecular Mass, M_r about 147,000, extracted from *Laminaria digitata*, containing 62% mannuronic acid and 38% guluronic acid) (high M/G ratio, NaA-H) was donated by Kelco International (Bagnolex Cedex, France). Sodium alginate (M_r about 115,000, extracted from *Laminaria hyperborea*, containing 30% mannuronic acid and 70% guluronic acid) (low M/G ratio, NaA-L) and chitosan (CHT) (low MW ≅ 70,000) were purchased from Fluka Chemie (Buchs, Switzerland). Tamoxifen, (Z)-2-[4-(1,2-diphenyl-1-butenyl)phenoxy]-N,N dimethylethylamine, citrate (TMX) was kindly donated by Solmag (Mulazzano, Lodi, Italy). All the other chemicals were of analytical grade and they were purchased from Carlo Erba (Milan, Italy).

2.2. Microparticle preparation

The microparticles were prepared by two steps, using both alginates (NaA-L and NaA-H).

2.2.1. Spray-drying step

TMX methanol solution (0.1%, w/v) was added to NaA-H or NaA-L aqueous solution (1%, w/v) and the 10/1 alginate/TMX mixture was spray-dried (Buechi 190 Mini-Spray Dryer, Buechi Laboratorium, Technik AG, Flawil, Switzerland) to obtain uncrosslinked microparticles in the following operating conditions: inlet temperature 140 °C, outlet temperature 60–65 °C, pump setting 5 ml min^{-1}, spray flow 600 Nlh^{-1}, aspirator setting 15 and nozzle cap diameter 0.5 mm.

2.2.2. Crosslinking step

The uncrosslinked microparticles were suspended in 10% (w/v) $CaCl_2$ aqueous solution (0.9 M), in a 0.1/1 microparticles/$CaCl_2$ ratio, under mechanical stirring (Ultraturrax, IKA, Labortechnik, Staufen, Germany) at 9000 rpm for 5 min. Subsequently, an equal volume of 1% (w/v) CHT, solubilized in pH 5.5 acetic acid solution, was added to the microparticle suspension under mechanical stirring and maintained for 10 min. The crosslinked microparticles were then recovered by centrifugation, rinsed with water and freeze-dried (Lyovac GT2, Leybold Heraues, GMBM, Köln, Germany).

2.3. Morphological and size analysis

Microparticle morphological structure was examined by a scanning electron microscope (SEM, XL-40, Philips, Eindhoven, The Netherland). Microparticle size was determined by computerized image analysis (IMG-VIEW, CIGS, University of Modena and Reggio Emilia, Italy) of at least 200 microparticles on SEM micrographs.

2.4. Zeta potential measurement

Zeta potential measurements of TMX loaded and unloaded crosslinked microparticles suspended in deionized distilled water were performed using a PALS zeta potential analyser (Ver. 3.29, Brookhaven Instruments Corp., Holtsville, NY).

2.5. Rheological analysis

To investigate the interaction occurring between alginates and TMX, rheological measurements were carried out on alginate/TMX water solutions by evaluating the reduced viscosity owing to an "electroviscous effect" produced by dilute solutions of polyelectrolytes. A 0.5% (w/v) solution of sodium alginate is considered a diluted polyelectrolyte solution (Stockwell et al., 1986). Therefore, rheological measurements were carried out by using both the sodium alginates and sodium alginate/TMX solutions, in a 10/1 ratio, prepared by mixing 1% (w/v) alginate solutions with an equal volume of 0.1% (w/v) TMX water solution containing 1% (w/v) Tween 80. After 24 h, the rheological behaviour was determined at 25 °C by placing 9 ml of the solutions in a coaxial cylinder (radii ratio = 1.02) rheometer (Rotovisco RV 12 Haake, Karlsruhe, Germany) and measuring the shear stress as a function of the shear rate. In the same conditions, a solution containing pure 0.05% (w/v) TMX was examined. The rheological experiments were carried out in triplicate.

2.6. Thermal analysis

Thermal analysis was performed on TMX in bulk, melted TMX, TMX in physical mixture with both alginates (10:1 alginate/TMX ratio), uncrosslinked and crosslinked microparticles. Thermograms were recorded on a differential scanning calorimeter (DSC-4, PerkinElmer, Norwalk, CT, USA) coupled with a computerized data station (PerkinElmer). Samples were heated in crimped aluminium pans at a scanning rate of 10 °C/min using nitrogen flow (30 ml min^{-1}).

2.7. FT-IR spectrophotometry

FT-IR analysis was performed on TMX in bulk, melted TMX, TMX in a physical mixture with both alginates (10/1 alginate/TMX ratio), uncrosslinked and crosslinked microparticles. An FT-IR spectrophotometer (Model FT-IR 1600, PerkinElmer) was used for recording the spectra of the samples in nujol mull.

2.8. TMX content determination

Microparticle TMX content was determined by dissolving exactly weighed amounts of microparticles in 6% (w/v) sodium citrate aqueous solution containing 1% (w/v) Tween 80 enhancing TMX solubility. Such a medium was selected because it contains calcium chelating ions as well as ions displacing the drug associated to alginate. After centrifugation at 12,000 rpm for 10 min, TMX concentrations in the supernatant were assayed spectrophotometrically (λ = 278 nm) (Lambda 35, PerkinElmer) in comparison with standard solutions. The reported data were averaged on three determinations and statistically analyzed by ANOVA one-way.

2.9. TMX in vitro release

TMX release from microparticles was performed under sink condition for 1 h in saline solution at pH 3.0 and for 2 h in simulated intestinal fluid at pH 7.4 containing 1% (w/v) Tween 80, enhancing TMX water solubility (0.5 mg/ml) (Memisoglu-Bilensoy et al.,

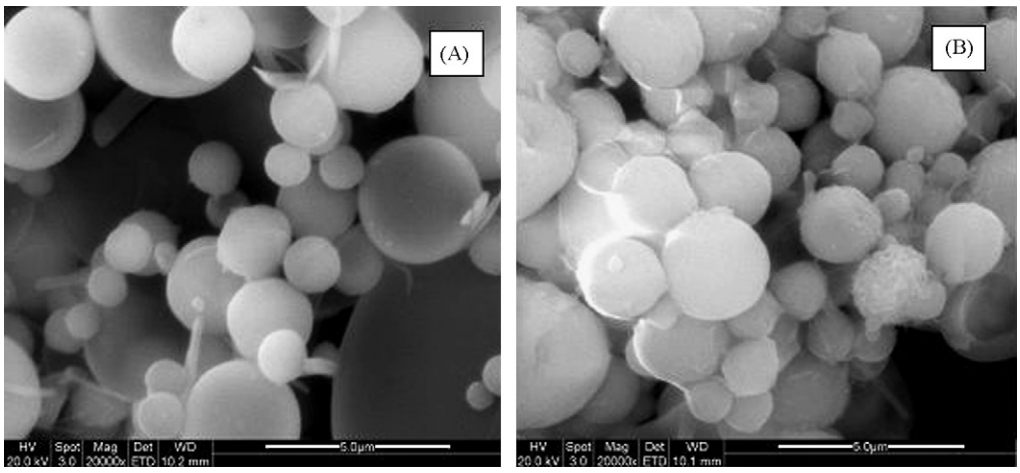

Fig. 1. SEM micrographs: (A) uncrosslinked microparticles and (B) crosslinked microparticles.

2005), under mechanical stirring (50 rpm) at 37 °C. After centrifugation, the concentration of the released drug was determined spectrophotometrically ($\lambda = 278$ nm) in the supernatant at fixed time intervals. The reported data were averaged on three determinations.

3. Results and discussion

Alginate microparticles loaded with TMX were developed by spray-drying technique and crosslinking by calcium ions and CHT. The parameters selected for the microparticle preparation resulted from previous preformulative works aiming to obtain microparticles with proper morphology, size, stability in gastro-intestinal fluids, drug loading level and release (Coppi et al., 2001, 2004).

3.1. Morphology, size and surface charge analysis

Spray-drying process of sodium alginate/TMX solutions led to uncrosslinked microparticles with nearly spherical shape (Fig. 1A) and size less than 3 μm with the most population (about 90%) in the range between 0.5 and 3 μm, regardless the alginate M/G ratio (Fig. 2) (Table 1). The crosslinking procedure did not modify significantly microparticle shape (Fig. 1B) and size distribution (Fig. 2) (Table 1). Microparticle shape and size could be considered proper for PP uptake and GALT delivery. In fact, microparticle size less than 3 μm is preferable because particles in the range of 3–10 μm do not migrate into mesenteric lymph nodes whereas too small particles can present difficulties with initiating phagocytosis (Florence, 1997). Zeta potential value was found +21.27 ± 2.45 mV for the TMX loaded microparticles and +21.91 ± 1.12 mV for the unloaded microparticles, regardless the alginate M/G ratio. Since most epithelial cells carry a net negative charge, a positively charge is expected to be more easily associated and taken up by the membranes (Andrianov and Payne, 1998).

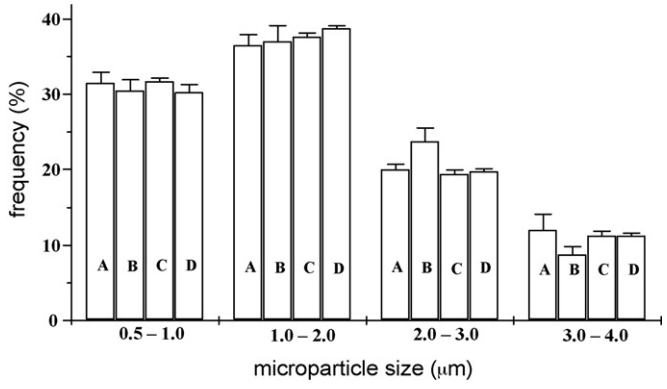

Fig. 2. Microparticle size distribution: (A) uncrosslinked Na-L/TMX, (B) uncrosslinked Na-H/TMX, (C) crosslinked Na-L/TMX and (D) crosslinked Na-H/TMX.

3.2. Rheological analysis

Complex formation between the polyanionic alginate and the oppositely charged TMX, having a cationic group, was examined by rheological analysis to evaluate the electroviscous effect of dilute polyelectrolyte solutions showing increased viscosities. This results from a spatial expansion of the hydrodynamic volume of the molecules due to electrostatic repulsions between charged segments subsequent to the dilution of the counter ion layer surrounding the molecules. In such conditions, the addition of oppositely charged compounds decreases the intramolecular repulsion leading to a tighter configuration and, consequently, to a reduced viscosity (Stockwell et al., 1986).

Since the rheological flow behaviour of TMX water solution corresponded to that of the medium, the rheograms of alginate/TMX solutions could represent the rheological properties of the polymer alone. The flow behaviour of NaA-H/TMX and NaA-L/TMX solutions are reported as shear stress in function of shear rate (Fig. 3). All the solutions showed a pseudo-plastic behaviour without hysteresis loops indicating that there was no change in the structure of the molecules under shear stress. The presence of TMX led to a slightly reduced viscosity of both the alginate solutions revealing that an electrostatic interaction between drug and alginate had occurred. Although cationic drugs are described as interacting preferentially with alginate mannuronic residues (Bystricky et al., 1990; Iannuccelli et al., 1996), the rheograms were not affected by the alginate M/G ratio probably owing to the low TMX association capacity.

Table 1
Microparticle size.

Microparticle sample	Mean diameter (μm)
Uncrosslinked Na-L/TMX	1.67 ± 0.86
Uncrosslinked Na-H/TMX	1.65 ± 0.83
Crosslinked Na-L/TMX	1.70 ± 0.76
Crosslinked Na-H/TMX	1.66 ± 0.81

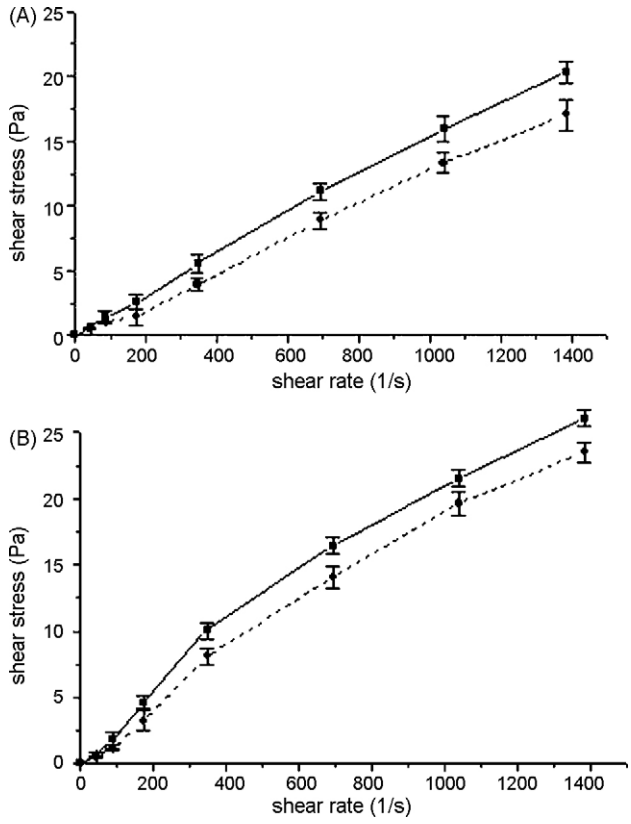

Fig. 3. Flow behaviour of water solutions containing 1% Tween 80: (A) NaA-L (solid line) and NaA-L/TMX (dash line) and (B) NaA-H (solid line) and NaA-H/TMX (dash line).

3.3. Thermal analysis and FT-IR spectrophotometry

TMX has been reported to exist in two polymorphic forms, A and B (Goldberg and Becker, 1987; Kojima et al., 2007). Thermogram of TMX in bulk shows an endothermic peak at 147 °C, corresponding to the melting point of TMX form A (Kojima et al., 2007). TMX melting endothermic peak, in the physical mixture with sodium alginate, was found lower (144 °C) owing to the impure form A (Fig. 4). The DSC curves of both uncrosslinked and crosslinked microparticles as well as that of melted TMX do not present endothermic peaks, suggesting that TMX is present as molecularly dispersed form in the alginate matrix.

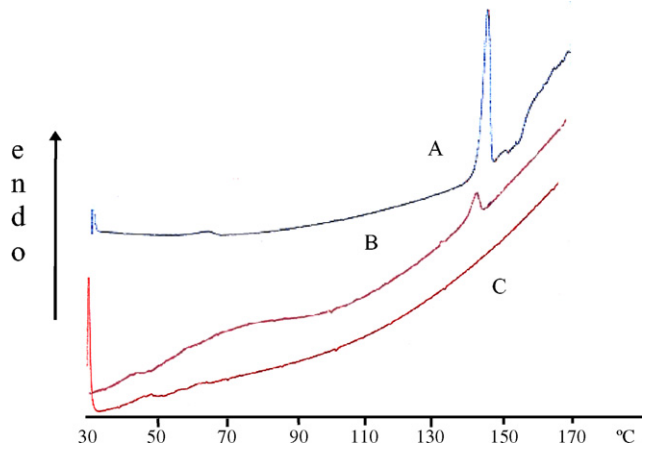

Fig. 4. DSC thermograms: (A) TMX in bulk, (B) NaA-L/TMX and NaA-H/TMX physical mixtures and (C) melted TMX, NaA-L/TMX and NaA-H/TMX uncrosslinked and crosslinked microparticles.

The FT-IR spectra (Fig. 5) of TMX in bulk, melted TMX and TMX in physical mixture with alginate present the characteristic band at 1731 cm^{-1} attributable to the vibrational stretching of C=O bond of citric acid involved in cyclic pairs of hydrogen bond interaction between adjacent citrate species as well as between citric carboxylate and TMX aminic group (Goldberg and Becker, 1987). Such a peak disappeared in the spectra of both uncrosslinked and crosslinked microparticles indicating a dissociation of the hydrogen bonds. This finding could not be related to the disruption of TMX crystal lattice since the stretching of C=O bond is present in

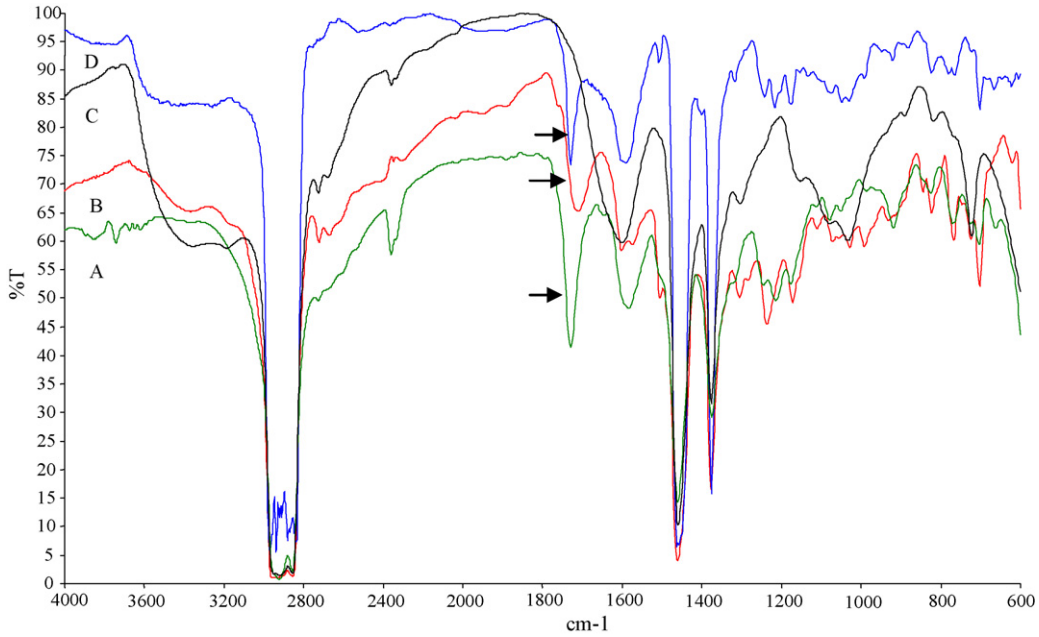

Fig. 5. FT-IR spectra (the arrows indicate the peak at 1731 cm^{-1}) (A) TMX in bulk, (B) melted TMX, (C) uncrosslinked and crosslinked NaA-L/TMX and NaA-H/TMX microparticles and (D) NaA-L/TMX and NaA-H/TMX physical mixtures.

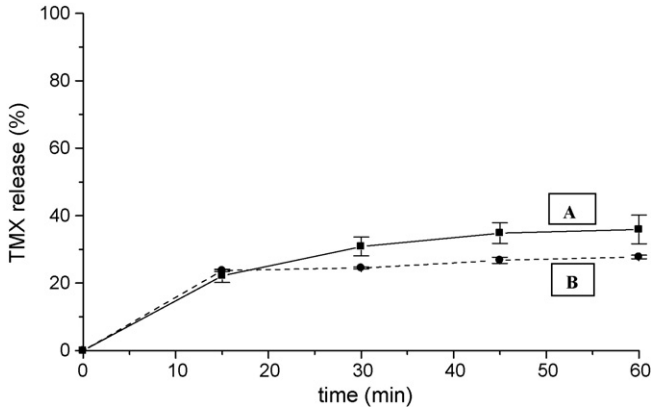

Fig. 6. *In vitro* TMX release in pH 3.0 medium: (A) crosslinked NaA-L/TMX microparticles (solid line) and (B) crosslinked NaA-H/TMX microparticles (dash line).

melted TMX. Therefore, the involvement of an interaction between TMX and sodium alginate could be supposed.

No effect on both thermal and FT-IR patterns was produced by changing the alginate M/G ratio.

3.4. TMX content

TMX amounts obtained by dissolving microparticle samples in sodium citrate solution could be considered as the total drug loaded into the microparticles. Drug loading levels were found $8.44 \pm 0.33\%$ (w/w) for NaA-H microparticles and $6.63 \pm 0.64\%$ (w/w) for NaA-L microparticles, with an encapsulation efficiency of 92.85 ± 3.68 and $72.93 \pm 7.11\%$, respectively, being the difference statistically significative ($p < 0.05$). Since the uncrosslinked microparticles contained $8.90 \pm 0.30\%$ (w/w) of TMX, with an encapsulation efficiency of $98.20 \pm 1.91\%$, regardless M/G ratio, a drug fraction leaching out from the microparticles during the crosslinking process could be presumed. Such a phenomenon was more relevant for the microparticles obtained by using an alginate having a low mannuronic acid content (NaA-L). By considering that TMX low association capacity was not affected by the alginate M/G ratio, the higher drug diffusion during the crosslinking process and, consequently, the lower drug loading level for NaA-L microparticles, could be attributable to the more porous structure of the low-M-rich gel network compared to the more entangled structure of the high-M-rich gels (Stokke et al., 2000).

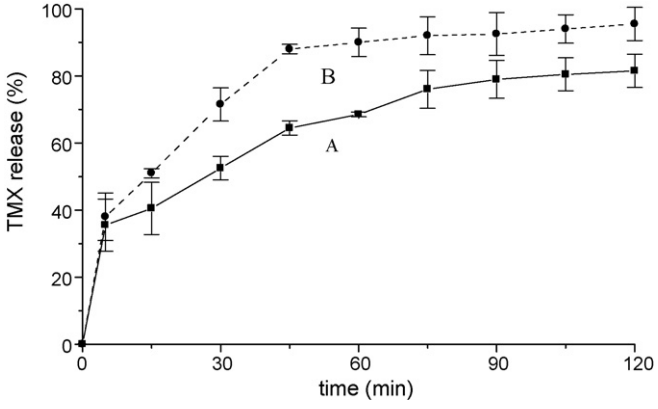

Fig. 7. *In vitro* TMX release in pH 7.4 medium (A) crosslinked NaA-L/TMX microparticles (solid line) and (B) crosslinked NaA-H/TMX microparticles (dash line).

Table 2
Values of kinetics exponent (n) of TMX release from the microparticle samples in gastro-intestinal media.

Release medium pH value	Alginate	n
3.0	Na-L	0.36 ± 0.05
3.0	Na-H	0.12 ± 0.02
7.4	Na-L	0.29 ± 0.02
7.4	Na-H	0.31 ± 0.03

3.5. TMX in vitro release

Microparticles were evaluated for TMX *in vitro* release in media at pH 3.0 (Fig. 6) and pH 7.4 (Fig. 7) in order to simulate the gastro-intestinal transit.

TMX release in acidic medium from the microparticles obtained by both the alginate types was sustained probably due to a restricted alginate neutralization that could determine a partial displacement from the complex.

The release data, studied in function of the power law (Korsmeyer et al., 1983) $M_t/M_\infty = kt^n$, where M_t/M_∞ denotes the drug fraction released at time t, k and n being the rate constant and kinetic exponent of release, respectively. The value of kinetic exponent n, defining the mechanism of the release process (Sinclair and Peppas, 1984), indicated diffusion type kinetics irrespective of the alginate type (Table 2).

TMX release in pH 7.4 medium from microparticles obtained by using NaA-H (Fig. 6) showed a triphasic profile, with a first burst phase in which about 40% TMX was delivered, a second fast phase leaching until 80% drug followed by a third sustained phase. TMX release from microparticles obtained by using NaA-L (Fig. 6) provided an initial burst phase in which about 40% drug was released in 15 min, followed by a second sustained phase.

The burst phase is presumably attributable to the release of superficial TMX, whereas the successive phases would be strictly related to the polymer material undergoing to breakage by non-gelling ions contained in the simulating intestinal medium. The slower drug diffusion rate from the microparticles obtained by using NaA-L could be reasonably ascribed to the greater crosslinking degree of the alginate with high G block content, showing higher selectivity for Ca^{2+} ions and then more resistance.

The power law equation applied to the release data from microparticles in pH 7.4 medium indicated diffusion type kinetics regardless the alginate M/G ratio (Table 2).

By considering that drug targeting to the GALT should involve microparticle integrity in the gastro-intestinal lumen retaining the drug until the uptake zone and that orally administered microparticles taken up by Peyer's patches were detected in the lymph within 10–15 min after dosing in fasted conditions (Hussain et al., 2001), a fairly good TMX dose would be transported by the particulate carrier and reach the intestinal uptake site.

4. Conclusions

Tamoxifen plays a relevant role in the management of female breast cancer in both early and advanced stages of disease although the advantages of side effects, uncompleted bioavailability and drug resistance. In the development of alginate/chitosan microparticles for the tamoxifen lymphatic delivery following the uptake by the intestinal Peyer's patches, the preformulative study performed in the present study indicated the occurring of an interaction between the drug and alginate involving hydrogen bonds. Moreover, microparticle polymer network and, consequently, drug loading level and release, were affected by the alginate mannuronic/guluronic ratio. Since the TMX physicochemical properties

could affect its efficacy, safety and pharmacokinetics, *in vitro* experiments on cell cultures as well as *in vivo* experiments will be performed on TMX loaded microparticles.

Acknowledgements

This work was supported by a grant (PRIN 06) of the Ministero dell'Istruzione, dell'Università e della Ricerca, Rome, Italy.

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