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A multiple emulsion method to entrap a lipophilic compound into chitosan microspheres

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Abstract

A new method for preparation of chitosan microspheres loaded with an hydrophobic drug, ketoprofen, was developed. It is an emulsification/solvent evaporation process carried out in mild conditions and particularly useful for microencapsulation of thermally sensitive drugs. This method can be additionally combined to physical and chemical cross-linking in order to modulate drug release. Physical cross-linking was carried out by dry heating chitosan microspheres at fixed temperatures and for different times. Glutaraldehyde at different concentrations was used as the chemical cross-linking agent on microspheres constituted by different theoretical ketoprofen/chitosan ratio (1:2, 1:4, 1:6 w/w). Chitosan microspheres were morphologically characterized for shape, surface characteristics and size distribution; chitosan/ketoprofen interactions inside microspheres were investigated by differential scanning calorimetry and powder X-ray difractometry. Ketoprofen contents inside the microspheres and in vitro drug release profiles were also determined. © 1997 Elsevier Science B.V.

Keywords: Chitosan; Microsphere; Multiple emulsion; Ketoprofen

1. Introduction

Chitosans are linear polysaccharides with low toxicity, that are biodegradable and biocompatible (Hirano et al., 1990). They have been considered for various biomedical and pharmaceutical applications (Akbuga, 1995). In the area of drug delivery systems chitosans and their derivatives have been usefully employed in the preparation of drug loaded microparticles (Ohya et al., 1993; Hassan et al., 1992; Thanoo et al., 1992; Genta et al., 1995).

Chitosan microspheres or microcapsules have been prepared by: emulsion or multiple emulsion cross-linking, simple coacervation, complex coacervation, emulsion/solvent evaporation, spray-drying (Thanoo et al., 1992; Pavanetto et al.,

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1996; Chandy et al., 1993; Takahashi et al., 1990; Li et al., 1991; Genta et al., 1994). However, none of the above quoted methods fulfils all the requirements of microencapsulation when formulating chitosan microspheres loaded with a hydrophobic drug. To this purpose in a previous study we successfully employed a O/W/O emulsification/phase separation method promoted by a chemical cross-linking agent (Pavanetto et al., 1996).

Afterwards we focused the study on chitosan microsphere preparation methods to several purposes: (a) microencapsulation of thermally sensitive drugs; (b) exclusion of irritant agents (i.e. cross-linking agents) to obtain microspheres suitable, e.g. for ophthalmic administration. With this aim a novel multiple emulsion preparation method is proposed in this work. It is an emulsification/solvent evaporation process that can be carried out in mild conditions and without any chemical cross-linking agent; it can be additionally combined to physical and chemical cross-linking in order to modulate drug release.

Ketoprofen was chosen as the hydrophobic model drug.

2. Materials and methods

2.1. Materials

Chitosan, M_r 750 000, deacetylation degree 83.5% was purchased from Fluka (Fluka, Buchs, Switzerland). Ketoprofen was obtained from Carlo Erba (Carlo Erba, Milano, Italy). Tween 80 and Span 20 were supplied by Atlas (Atlas, Essen, Germany), 25% w/v glutaraldehyde aqueous solution was supplied by Aldrich (Aldrich Chemie, Steinheim, Germany). Methylene chloride, glacial acetic acid, methanol, mineral oil and petroleum ether (Carlo Erba, Milano, Italy) were of analytical grade. The distilled water used was prepared in our laboratory.

2.2. Microsphere preparation method

2.2.1. Chitosan microspheres

Chitosan microspheres have been prepared according to the 'dry-in-oil multiple emulsion' method, depicted in Fig. 1.

This technique was accomplished by three steps:

1. preparation of a primary o/w emulsion in which the 'oily dispersed phase' is constituted of CH_2Cl_2 and the 'aqueous continuous phase' is a mixture of 2% v/v CH_3COOH solution: methanol (4:1 v/v) containing the chitosan (1.6% w/v) and Tween 80 (1.6% w/v);
2. multiple emulsion formation with mineral oil ('oily outer phase') containing Span 20 (2% w/v);
3. evaporation of aqueous solvents at reduced pressure.

The primary o/w emulsion was obtained by injecting CH_2Cl_2 into the aqueous continuous phase; the ratio between the two phases of the emulsion was 1:2.5 v/v. Emulsification was performed at room temperature under continuous stirring (Ultraturrax model T25 S25N10G) at 9500 rpm for 15 min.

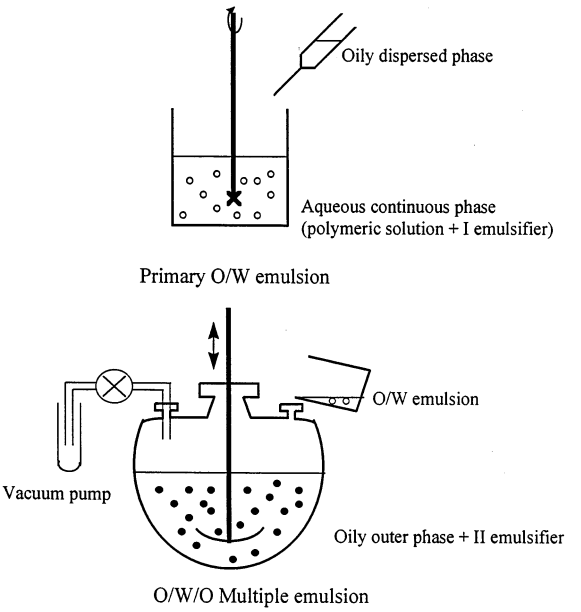

Fig. 1. Schematic procedure for preparation of chitosan microspheres by the 'dry-in-oil multiple emulsion' method.

The primary emulsion was dropped into mineral oil (1:9 v/v ratio), thermostated at 50°C and stirred by a vibromixer (Vibromixer E1 Chemap-AG, Volketswil, Switzerland) at 50 vibration/s. Pressure was reduced by a vacuum pump after 2 h and the system was warmed up to 60°C in 30 min; these conditions of temperature and pressure were maintained under continuous stirring for 19 h until the aqueous solvents were evaporated completely.

The microsphere suspension was rinsed three times with petroleum ether; the microspheres collected by centrifugation at 3000 rpm were filtered through a 2.5 μm membrane filter and dried under vacuum for 48 h.

Ketoprofen loaded chitosan microspheres were prepared as described above by dissolving the drug into CH_2Cl_2 constituting the dispersed phase. The batches of microspheres have been produced in triplicate.

2.2.2. Heat treated chitosan microspheres

Three batches of chitosan microspheres produced as described above were treated in an air oven at 80°C for 5 h and three batches for 21 h. Samples of ketoprofen underwent the same thermal treatment as the microspheres.

2.2.3. Chemically cross-linked chitosan microspheres

Chemically cross-linked chitosan microspheres were prepared by the 'dry-in-oil multiple emulsion' method, as described above, dropping the cross-linking agent i.g. glutaraldehyde aqueous solution into the multiple emulsion at the time of formation (1:5 v/v cross-linking agent solution: aqueous phase).

In these experiments the influence of ketoprofen concentration in the oily dispersed phase and of glutaraldehyde concentration was evaluated. All batches have been produced in triplicate. Table 1 lists all the batches of ketoprofen loaded chitosan microspheres produced.

2.3. Characterization

2.3.1. Morphology

2.3.1.1. Optical microscopy. The formation of microspheres was monitored during preparation steps by an optical microscope Laborlux Leitz model K (Leitz s.r.l., Milan, Italy) with transmitted light at 500 × magnification.

2.3.1.2. Scanning electron microscopy. The microspheres were characterized by scanning electron microscopy (SEM). An electron microscope Jeol JX 840-A (Jeol LTD, Tokyo, Japan) was used. Samples for SEM observation were prepared by the dried microspheres sputter-coated under argon atmosphere with a thin layer of Au/Pd.

2.3.2. Particle size analysis

Particle size was determined on samples of microspheres suspended in a 5% w/v NH_4SCN isopropanolic solution saturated with ketoprofen. The samples have been analyzed by a Coulter Counter Multisizer model TA II (Coulter Electronics, Luton, United Kingdom). The analyses were carried out at 64 size levels, between 1.792 and 58.96 μm. The results are the average of five analyses.

2.3.3. Differential scanning calorimetry (DSC)

DSC analyses were performed by a DSC Mettler model TA 3000 (Mettler, Zurich, Switzerland). Samples (5–6 mg) were scanned in aluminium pans, in a nitrogen atmosphere, in the 30–250°C temperature range, with a heating rate of 10°C/min.

2.3.4. Powder X-ray difractometry

Powder X-ray difractometry analyses were carried out using a Philips mod.1050 PW1730 (Philips, Eindhoven, Netherlands) difractometer with graphite monochromator Cu–K_α radiation. Samples blank microspheres, the physical mixture of blank microspheres and ketoprofen, ketoprofen and drug loaded microspheres were analyzed.

Table 1
Ketoprofen loaded chitosan microspheres

Batch no.	Ketoprofen solution (%)	Drug/chitosan (w/w)	Heat treatment (h)	Glutaraldehyde solution (%)	Actual drug content (%)	Encapsulation efficiency[a] (%)
1–3	2.0	1:2	—	—	3.72	11.16
4–6	2.0	1:2	5	—	3.65	10.95
7–9	2.0	1:2	21	—	3.73	11.19
10–12	2.0	1:2	—	1.25	7.09	21.26
13–15	2.0	1:2	—	2.50	8.07	24.20
16–18	2.0	1:2	—	5.00	8.05	24.15
19–21	1.0	1:4	—	5.00	6.23	31.16
22–24	0.65	1:6	—	5.00	4.85	34.02

[a]

$$\text{Encapsulation efficiency} = \frac{\text{Actual drug content}(\%)}{\text{Theoretical drug content}(\%)}$$

2.4. Microsphere drug content

The chitosan microspheres loaded with ketoprofen were dissolved in a HCl 0.1 N/ethanol (1:1 v/v) mixture. The amount of drug loaded was determined by analysis with an HPLC Varian model 9010 (Varian, Milano, Italy). A Lichrosorb RP18 column 25×0.4 cm (Merck Bracco, Milano, Italy) was employed in conjunction with a UV detector at 254 nm. The mobile phase was a methanol/0.04% CH_3COOH mixture (65:35 v/v). An ethanolic solution of ketoprofen (10 μg/ml) was employed as the external standard.

2.5. In vitro drug release studies

In vitro release tests were carried out on all batches of ketoprofen loaded chitosan microspheres.

Amounts of microspheres containing about 1 mg of ketoprofen, as well as a reference sample made of the same amount of drug, were suspended in 100 ml phosphate buffer pH 7.2 (USP XXIII) containing ethanol (10% v/v).

The microsphere suspensions contained in capped Erlenmeyer flasks were agitated in a shaker incubator (Isco Italia, Milan, Italy) at 30 St/min for 384 h at 37°C.

At scheduled time intervals, the agitation was stopped, the microsphere suspensions were allowed to settle for 5 min and 400 μl of the dissolution medium was collected and analyzed for drug content. Dissolution medium was replaced with fresh medium. All dissolution tests were run in triplicate and mean values reported.

3. Results and discussion

The novel 'dry-in-oil multiple emulsion' preparation method proposed for loading a lipophilic drug into chitosan microspheres is based on the formation of a O/W/O multiple emulsion in which the droplets of the primary O/W emulsion in the oily outer phase must be stable enough to permit the subsequent process of solvent evaporation, involving mild heating and vacuum application.

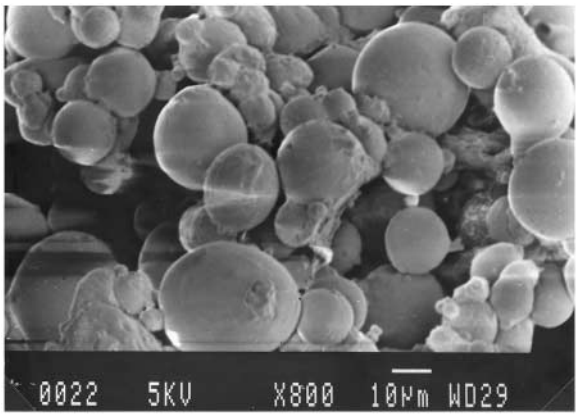

Fig. 2. Scanning electron micrograph of chitosan microspheres (batch 2).

In relation to the viscosity of the drug solutions and of the oily outer phase a 1.6% (w/v) polymeric solution resulted in the most stable emulsion; moreover methanol was added to aqueous acetic solution in order to improve the formation of spherical chitosan droplets in liquid paraffin (Lin et al., 1992).

During microsphere preparation the heating program was standardized so as to permit gradual evaporation of aqueous solvent without emulsion de-stabilization or aggregation of the newly formed polymeric phase.

The standardized operating conditions permitted the production of single, not aggregated chitosan microspheres in a reasonable period of time without addition of a chemical cross-linking agent.

The morphology of the microparticles produced was investigated by scanning electron microscopy. Fig. 2 shows a typical scanning electron micrograph of the microspheres (batch 2). The microspheres appear well-formed, with spherical and regular shape and smooth surface indicating the achieved optimization of solvent evaporation rate during formation process. Some batches of microspheres (Table 1, batches 4–9) were subjected to dry heating in an air oven: the heating temperature was chosen at 80°C because of the ketoprofen melting point Heat treatment on hydrophilic polymers, such as albumin or chitosan, induces the formation of cross-links be-

tween polymeric molecules and/or the formation of crystallites increasing the water resistance of the materials (Pavanetto et al., 1994; Lim et al., 1995) The degree of enhancement in water resistance of chitosan is induced both by temperature and duration of heat treatment (Lim et al., 1995). Ketoprofen loaded chitosan microspheres were subjected to heat treatment at 80°C for different times, 5 or 21 h in order to induce a different cross-linking degree.

Heat treatment did not change colour and morphologic characteristics of the microspheres nor the microstructure of chitosan microspheres as viewed under SEM (Fig. 3).

Chemical cross-linking of chitosan microspheres was obtained using a well-known chemical cross-linking agent, glutaraldehyde. It is known that the cross-linking mechanism involves formation of Schiff's base structures (Jayakrishnan et al., 1996). In the same reaction conditions (pH, times, temperatures and agitation), the cross-linking rate increases as more concentrated glutaraldehyde or chitosan solutions were used (Roberts et al., 1989). Cross-linked chitosan microspheres were prepared using a polymeric solution at a fixed concentration and glutaraldehyde solutions at different concentrations (1.25–2.5–5% w/v); time and temperature of cross-linking reaction were maintained constant (Table 1, batch no. 10–18). Moreover, batches of microspheres were formulated in the same cross-linking conditions and at different theoretical ketoprofen contents, by using drug solutions at different concentrations (Table 1, batch no. 16–24).

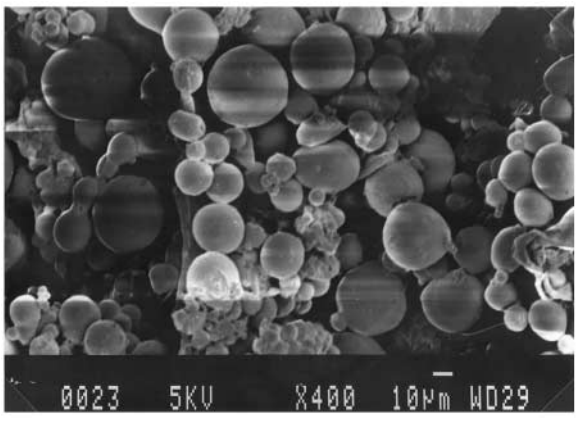

Fig. 3. Scanning electron micrograph of chitosan microspheres (batch 2, treated at 80°C for 24 h).

Table 2
$D_{v50\%}$ and $d_{v90\%}$ of some batches of chitosan microspheres by Coulter Counter analyses

Batch no.	$D^{a}_{v50\%}$ (μm)	$D^{b}_{v90\%}$ (μm)
1	19.81	31.35
16	10.73	15.61
19	12.67	20.50
22	12.18	19.11

[a] Fifty percent of the particles have a diameter lower than the tabulated value.
[b] Ninety percent of the particles have a diameter lower than the tabulated value.

SEM studies confirm that chemical cross-linking and above all the mentioned different experimental conditions have no influence on the morphological characteristics of microspheres. Furthermore, the use of glutaraldehyde at increasing concentrations has induced the formation of a yellow-brown colour more intense as a function of the higher cross-linking density (Roberts et al., 1989).

All batches of microspheres were analyzed by Coulter Counter for size distribution. In Table 2 $d_{v50\%}$ e $d_{v90\%}$ of more significant batches of microspheres are reported. A correlation between microsphere size and cross-linking agent concentration was observed. Particle size analyses show that glutaraldehyde addition decreased microsphere sizes, particularly for 5% w/v glutaraldehyde solutions and for microspheres with 1:2 theoretical drug/polymer ratio.

Physico-chemical characterization of microspheres was carried out by DSC and powder X-ray analyses.

DSC analyses did not provide information on the microstructure of the microparticles because chitosan and ketoprofen present endothermic bands approximately at the same temperature, 96°C and about 110°C respectively, so that it is impossible to discriminate their thermical behaviour when they are simply mixed together or formulated as microspheres.

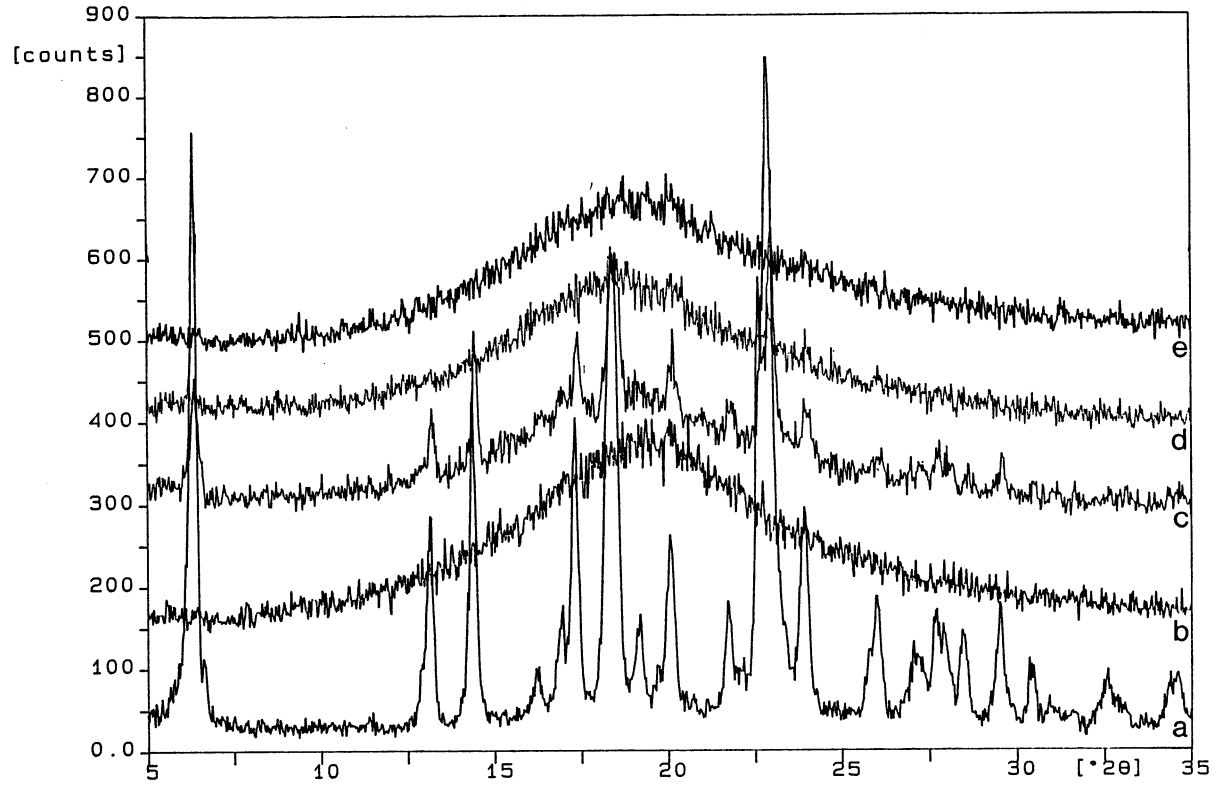

Fig. 4. Powder X-ray patterns of: (a) ketoprofen; (b) chitosan; (c) ketoprofen/chitosan physical mixture containing 33% of ketoprofen; (d) ketoprofen/chitosan physical mixture containing 8% of ketoprofen; and (e) ketoprofen loaded chitosan microspheres (batch 18).

X-ray analyses were performed on ketoprofen, chitosan, physical mixtures of ketoprofen and chitosan in which ketoprofen concentration was 33% or 8% w/w and ketoprofen loaded microspheres (batch 18). The relative X-ray difraction patterns are reported in Fig. 4. Ketoprofen shows a typical pattern of crystalline substance (Fig. 4(a)) and chitosan that of amorphous material (Fig. 4(b)). Ketoprofen loaded microparticles do not reveal any significant peak attributable to the crystalline drug, but only to apparently amorphous components (Fig. 4(e)), the same appears for chitosan/ketoprofen physical mixture at 8% w/w drug concentration (Fig. 4(d)).

Even, the physical mixture containing 33% of ketoprofen, corresponding to the theoretical drug content in microspheres, shows a pattern of partially amorphous drug (Fig. 4(c)).

These results suggest that the drug is amorphous inside the microspheres and that also a physical mixing of chitosan and drug affects the ketoprofen lattice structure.

Table 1 reports ketoprofen content in the different batches of microspheres and relative encapsulation efficiencies (each value reported is the mean of three batches data). These data show that heat treatment did not affect microsphere drug content and chemical cross-linking by glutaraldehyde can increase encapsulation efficiency by about 50% (from about 11 to 24%) when drug/polymer ratio is kept constant (1:2, w/w); higher ketoprofen content was achieved using glutaraldehyde solutions at concentrations equal or higher than 2.5% w/v. Ketoprofen/chitosan ratio influences drug encapsulation efficiency: the highest drug encapsulation efficiency was reached by the lowest ratio 1:6 w/w. Yields of

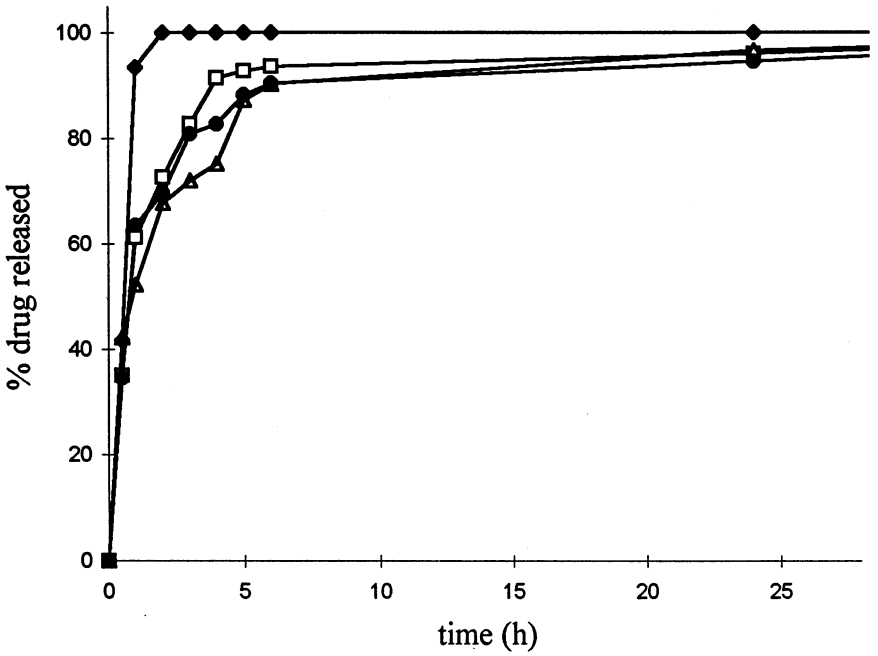

Fig. 5. Dissolution profiles of ketoprofen released from: □, batch 1; ●, batch 4; △, batch 7; and ◆, ketoprofen (raw drug).

process were always very satisfactory (higher than 75%).

The influence of the different cross-linking methods on ketoprofen release from chitosan microspheres was evaluated by in vitro dissolution tests.

In vitro ketoprofen release profile from the different batches of microspheres are shown in Figs. 5–7. Fig. 5 shows the dissolution profiles of batch 1, untreated or heated at 80°C for 5 or 21 h. Heat treatment did not induce significant changes in drug release rate from microspheres: as shown in Fig. 5 about 90% of drug has been released after 6 h from all batches.

Slight differences in the dissolution profiles of batches 1, 4 and 7 can be observed within the first 6 h: release rate of drug from batch 7 appears to be slower (about 70% in 4 h). It can be suggested, as an explanation, that the extended heat treatment (21 h) generates a surface cross-linked layer effective in slowing down drug release rate in the first 4 h.

Fig. 6 shows ketoprofen dissolution profiles from microspheres prepared with the same theoretical drug/polymer ratio (1:2) and treated with solutions of glutaraldehyde at different concentrations. As expected, higher glutaraldehyde concentrations induced more dense polymer cross-linking responsible of slower release of drug: after 24 h, uncross-linked microspheres have released 95.9% of encapsulated drug while 83.2, 66.4 and 49.1% of total drug content have been released respectively from microspheres cross-linked with 1.25, 2.5 and 5% w/v glutaraldehyde solutions.

Fig. 7 shows the dissolution profiles of ketoprofen from chitosan microspheres with different drug/polymer ratios (1:2, 1:4 and 1:6 w/w) and same cross-linking density.

In these conditions the amount of polymer seems to slow down drug dissolution rate from microspheres: in fact after 24 h only 25% of drug loaded was released from the microspheres with the lowest drug/polymer ratio.

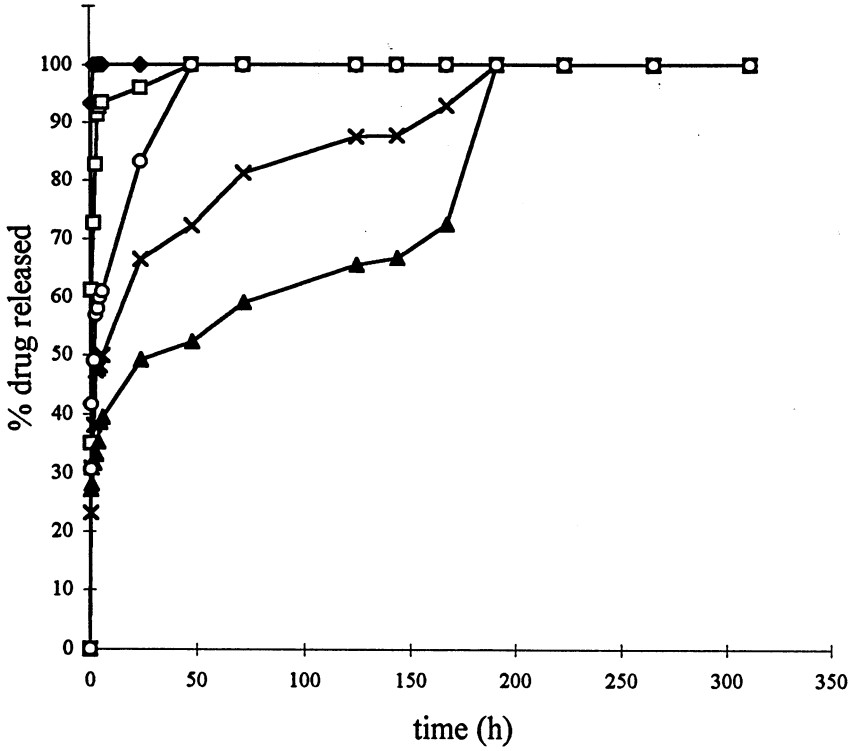

Fig. 6. Dissolution profiles of ketoprofen released from: □, batch 1; ○, batch 11; *, batch 14; ▲, batch 18, of ◆, ketoprofen (raw drug).

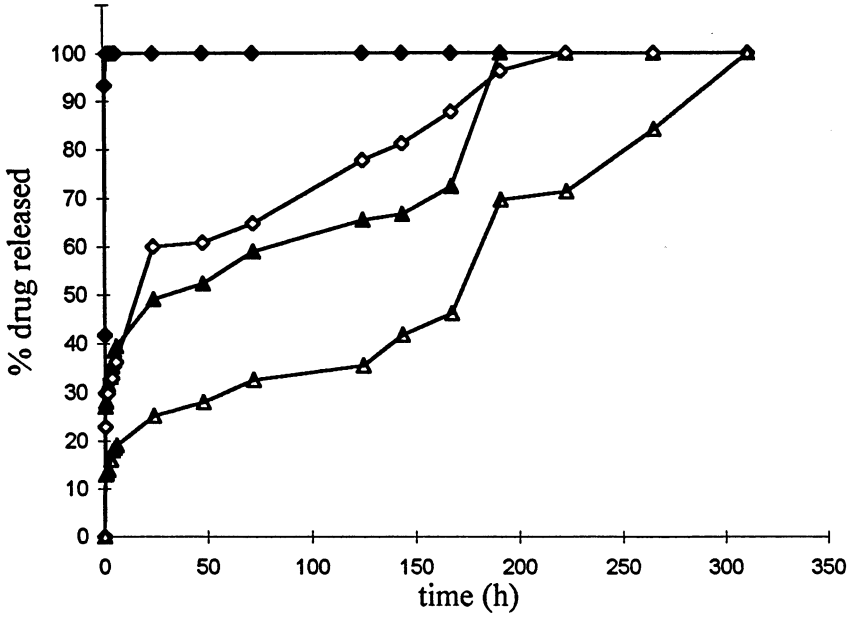

Fig. 7. Dissolution profiles of ketoprofen released from: ▲, batch 18; ◇, batch 20; △, batch 24; and ◆, ketoprofen (raw drug).

4. Conclusions

The multiple emulsion method proposed for the preparation of chitosan microspheres was found to be a good technique to encapsulate hydrophobic drugs in hydrophilic polymers.

Chitosan microspheres with good morphological characteristics and narrow size distribution have been prepared without any cross-linking agent addition.

The properties of the microparticles can be modified by physical and chemical polymer cross-linking. Among these treatments chemical cross-linking with glutaraldehyde proved to be more suitable to obtain modulation of ketoprofen release. To achieve a desired drug release rate, a suitable ketoprofen/chitosan ratio and glutaraldehyde concentration should be used.

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