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Radical Crosslinked Albumin Microspheres as Potential Drug Delivery Systems: Preparation and In Vitro Studies

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This article reports on the preparation of acryloylated bovine serum albumin microspheres and the evaluation of their employment in drug delivery areas. The influence of preparation parameters on albumin microspheres and the chemicophysical properties of loaded drugs were investigated. In particular, we focussed on acylation albumin degree and the amount of acryloylated albumin against comonomer in the polymerization step. Finally the release profile took into consideration the interaction drug-matrix, the fuctionalization degree of albumin, and the water affinity of matrix.

Keywords Acryloylated Bovine Serum Albumin, Spherical Microparticles

Considerable interest in recent years has been shown in the use of microspheres as a carrier system for drug delivery (Truter, Santos, Els 2001; Muzzalupo et al. 2001; Fundueanu et al. 2001; Miyata, Uragami, Nakamae 2002; Madhan Kumar and Panduranga 1998). Polymer based on microspheres have been widely studied for drug controlled release. It is known that synthetic polymeric materials are applied for their moldable properties, such as molecular weight and crosslinked degree, but they do not exhibit biodegradability and biocompatibility. Naturally occurring polymers could avoid toxicity or biodegradability problems related to the use of synthetic materials (Majors and Friedman 1991; Kao et al. 2003; Gallo and Gupta 1989; Widder and Seneyei 1983). In particular albumin is an attractive macromolecular carrier used to prepare microspheres in the large size range extensively used for sustained delivery of therapeutic agents and as drug carriers. These beads are used to realize site-specific deliver systems able to influence the remote site release of drugs.

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By virtue of its ability to interact with a wide variety of drugs and its simple and low cost preparation, albumin represents a very interesting material for therapeutic applications. A considerable number of strategies have been developed to obtain albumin microspheres. They can be achieved by thermal denaturation either by direct reaction between functional groups (usually carboxyl and amino goups) in the polypeptide side chains and also by chemically crosslinking agents such as bifunctional carbonyl reagents (Merodio et al. 2001; MacAdam et al. 1997; Sahin et al. 2002). Microspheres have been obtained from bovine serum albumin not denatured (Longo et al. 1982; Katti and Krishnamurti 1999). All the techniques employed in the past carried out hydrophobic materials able to release the drug through an erosion mechanism.

In a previous article we reported on the preparation of hydrogels via radical copolymerization reaction employing as starting material the copolymer PHEA-GMA obtained by partial derivatization of a polyamino acid such as α,β-poly-(N-2-hydroxyethyl)-DL-aspartamide (PHEA) with glycidylmethacrilate (GMA) (Muzzalupo et al. 2001; Pitarresi et al. 2001; Pitarresi et al. 2004). More recently we have reported on the preparation of hydrogels, with the same procedure, from partially methacrylated albumin based (BSA-Ma) (Iemma et al. 2004). The functionalization of PHEA with GMA and BSA with methacrylic anhydride (Ma), respectively, allowed us to introduce reactive acryilic groups in the side chain to facilitate radical reactions and to obtain biodegradable hydrogels.

The objectives of this study are twofold: (1) production and characterization of BSA-Ma microspheres designed for oral formulation and (2) the release characteristics of drug from microspheres. For our purpose, we have chosen as a model diflunisal (DF), β-propranolol (PP), and 5-fluorouracil (FU). Such drugs have been selected to evaluate their in vitro release profile under conditions mimicking gastrointestinal fluid in relation to their different chemical properties. Moreover, because of the chemical diversity of drugs, we made careful selection of a loading technique with matching properties. All hydrogels obtained were characterized by particle size distribution analysis, scanning electronic microscopy, and swelling behavior. In vitro release studies, in simulated gastrointestinal fluids, showed the

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influence of the environmental pH, of beads composition, and of the chemical nature of entrapped drug.

MATERIALS AND METHODS

All the reagents used were of analytical grade, unless otherwise stated n-hexane and carbon tetrachloride, purchased from Aldrich Chemical Co., were purified by standard procedures. N,N-dimethyilacrylamide (DMAA), methacrylic anhydride (Ma), 2,4,6-trinitrobenzensulphonic acid (TNBS), sorbitan trioleate (Span 85), polyoxyethylene sorbitan trioleate (Tween 85), N,N,N′,N′-tetramethylethylendiamine (TMEDA) and ammonium persulfate were bought from Fluka Chemical Co. 5-fluorouracil, diflunisal, and propranolol were provided by Aldrich Chemical Co.

BSA-Ma was prepared according to a procedure elsewhere reported. BSA fraction V (MW 68.000; pH 7.0 ± 0.2; grade ≥98%) was from Roche Diagnostics GmbH. Derivatization of BSA with Ma to produce BSA-Ma (A and B) was carried out in distilled aqueous phase, under conditions of controlled pH and temperature (pH7 and 0°C), using a suitable amount of Ma and stirred for 1 hr, purified and characterized following the procedure reported elsewhere (Iemma et al. 2004). The derivatization degree (DD) of prepared BSA-Ma was determined in agreement with a procedure reported in literature (Snyder and Sobocinski 1975). The beads were newly prepared to have fresh and greater amounts of materials.

Apparatus

The dialysis tubes used are 6-27/32″ (Medicell International LTD). Freezing-drying apparatus was Micro Modulyo, (Edwards). Ultraviolet (UV) spectra were recorded with a U-2000 Hitachi spectrophotometer using 1 cm quartz cells. The number of scans was 100. High pressure liquid chromatography (HPLC) analyses were carried out using a Jasco PU-2080 liquid chromatography equipped with a Rheodyne 7725i injector (fitted with a 10 μl loop), a Jasco UV-2075 HPLC detector, and Jasco-Borwin1 integrator. A reversed-phase C18 column (μ Bondapak, 10 μm of 250 × 4.6 mm internal diameter obtained from Waters) was used. Particle size distribution was carried out using an image processing and analysis system, Leica DMRB equipped with a Leica Wild 3D stereomicroscope. This image processor calculates the particle area and converts it to an equivalent circle diameter. Scanning electron microscopy (SEM) photographs were obtained with a Leo stereoscan 420; the sample surface was made conductive by the deposition of a layer of gold on the samples in a vacuum chamber.

Microspheres Preparation (Standard Procedure)

Microspheres BSA-Ma based were produced by radical copolymerization technique previously described. Briefly, a mixture of n-hexane and carbon tetrachloride was placed in a round-bottom cylindrical glass reaction vessel fitted with an anchor-type stirrer and thermostated at 40°C, then treated, after 30 min of N_2 bubbling, with a solution of BSA-Ma, comonomer (DMAA), and ammonium persulfate in water. The density of the organic phase was adjusted by the addition of CCl_4 or n-hexane so that the aqueous phase sank slowly when stirring stopped. Under stirring at 1000 rpm, the mixture was treated with Span85 and Tween85, then after 10 min with TMEDA, and stirring was continued for another 60 min. The amounts of all reagents used in these experiments are reported in Table 1. Each matrix so obtained was filtered; washed with 50 ml portions of 2-propanol, ethanol, and acetone; and dried overnight under vacuum at 40°C.

Water Content of Microspheres

The swelling characteristics of BSA-Ma (A_{1-2} and B_{1-2}) microspheres were determined to check hydrophilic affinity of spherical microparticles. Typically aliquots (40–50 mg) of the microparticles dried to constant weight were placed in a tared 5-ml sintered glass filter (Ø10 mm; porosity, G3), weighted, and left to swell by immersing the filter plus support in a beaker containing the swelling media: double distilled water, HCl 0.1 N (simulated gastric fluid), and phosphate buffer pH 6.8 (simulated intestinal fluid). At a predetermined time, the excess water was removed by percolation at atmospheric pressure. Then, the filter was placed in a properly sized centrifuge test tube by fixing it with the help of a bored silicone stopper, then centrifuged at 3500 rpm for 15 min, and weighted. This operation was repeated at different times (1, 4, and 24 hr). The filter tare was determined after centrifugation with only water. The weights recorded at the different times were averaged and used to give the water regain by the following equation:

$$WR(\%) = \frac{W_s - W_d}{W_s} \times 100 \quad [1]$$

Where W_s and W_d are weights of swollen and dried spherical microparticles, respectively (Table 2). Each experiment was carried out in triplicate and the results were in agreement within ±4% standard error.

TABLE 1
Homopolymerizations and copolymerizations with DMAA of derivatizated BSA

Aqueous dispersed phase		Organic continous phase	Resin	
BSA-Ma mg/DD%	DMAA mmoli/mg	CCl_4/Esano ml/ml	mg (conv. %)	Initial
350/63	2.23/221	16/23	513 (90)	A_1
450/63	0.45/45.1	15/23	376 (76)	A_2
350/100	2.23/221	16/23	560 (98)	B_1
450/100	0.45/45.1	15/23	416 (84)	B_2

For all the polymerization aqueous phase amount is 3 ml; initiator system is $(NH_4)S_2O_8$/TMEDA (100 mg/150 μl); surfactants are Span85/Tween 85 (120 μl/30 μl).

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TABLE 2
Water regain percent (WR%) of beads in various media

	Water regain percent		
Sample	H_2O	pH1	pH6.8
A_1	235	186	237
A_2	130	102	194
B_1	200	197	282
B_2	149	122	249

Incorporation of Drug into Preformed BSA-Ma Microspheres

Incorporation of drugs into preformed BSA-Ma microspheres was performed as follows: 150 mg of preformed empty microspheres (prepared as described above) were wetted with 2 ml in a concentrated drug solution (15 mg/ml). After 3 days, under slow stirring at room temperatures, the microspheres were freed of the solvent at reduced pressure in presence of P_2O_5 to constant weight. The weights of drugs were determined by the difference between loaded matrix weight and the empty matrix one, respectively.

In Vitro Drug Release at pH 1.0 and 6.8 from Microparticles

In vitro drug release profiles were obtained by HPLC. Aliquots (10 mg) of drug-loaded BSA-Ma microparticles were dispersed in flasks containing HCl 0.1 N (pH 1.0, simulated gastric fluid) and maintained at 37 ± 0.1°C in a water bath for 2 hr with magnetic stirring. After this time, a solution of 0.2 M tribasic sodium phosphate was added to raise the pH to 6.8 (simulated intestinal fluid), according to the method reported in USP XXII (drug release test, method A, for enteric-coated particles). Sink conditions were maintained throughout the experiment. Then at suitable time intervals, samples were filtered and the solutions were analyzed by HPLC. Each experiment was carried out in triplicate and the results were in agreement within ±5% standard error.

RESULTS AND DISCUSSION

The reaction of BSA with Ma in water at 0°C and neutral pH allows us to obtain BSA methacrylated samples with 63% and 100% of acylated available amino groups, A and B, able to take part in a radical polymerization for the preparation of microspheres. A and B, respectively, were crosslinked by radical polymerization through a reverse-phase suspension polymerization technique in the presence of a comonomer (DMAA) and using TMEDA and ammonium persulfate as initator systems (Table 1).

A_{1-2} and B_{1-2} microparticles were perfectly spherical (Figures 1a–1d) and show a narrow size distribution (Figures 2a–2d). To evaluate the affinity of prepared beads toward aqueous medium, the value of contained water percentage (WR%) was determined in aqueous media that simulate some biological fluids, such as gastric (pH 1) and intestinal (pH 6.8) liquid and also in distilled water. The water regain percent, summarized in Table 2, demonstrate that albumin-based microspheres swell in an aqueous environment due to hydration.

The extent of swelling process depends on composition of reaction mixture. Therefore with higher DMAA concentration, the beads are more hydrophilic. The values of WR% suggest that the swelling capacity decreases in the order $A_1 > A_2$, such as $B_1 > B_2$, in accord with the increase in the amount of DMAA in the sample. However, there was not a remarkable difference

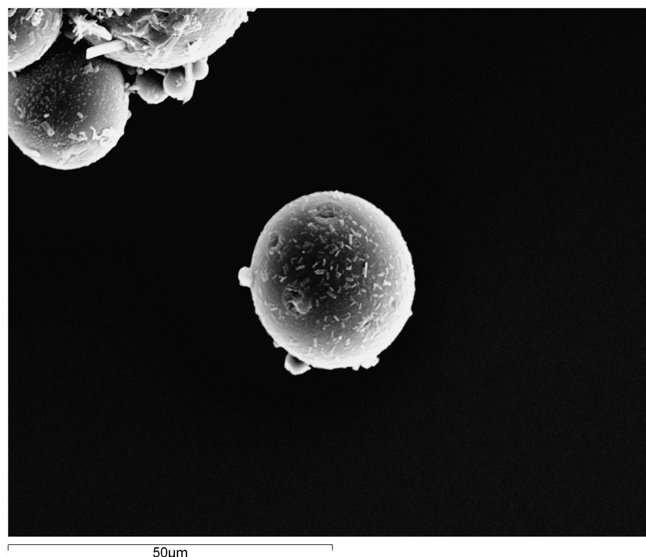

FIG. 1a. SEM micrograph of A_1.

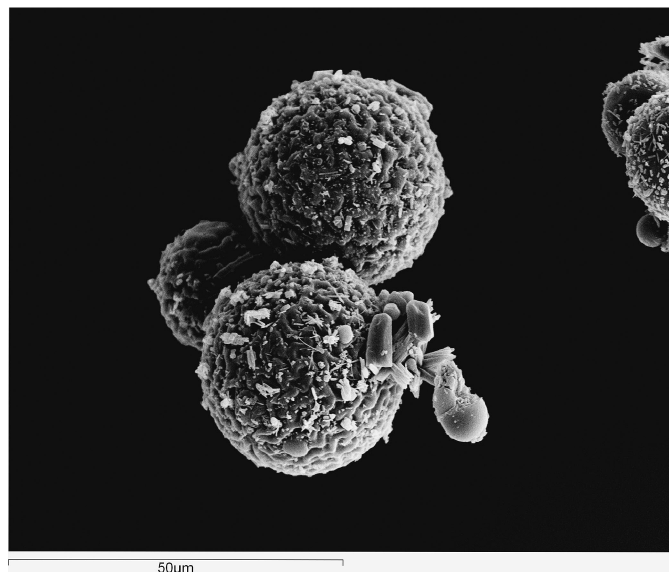

FIG. 1b. SEM micrograph of A_2.

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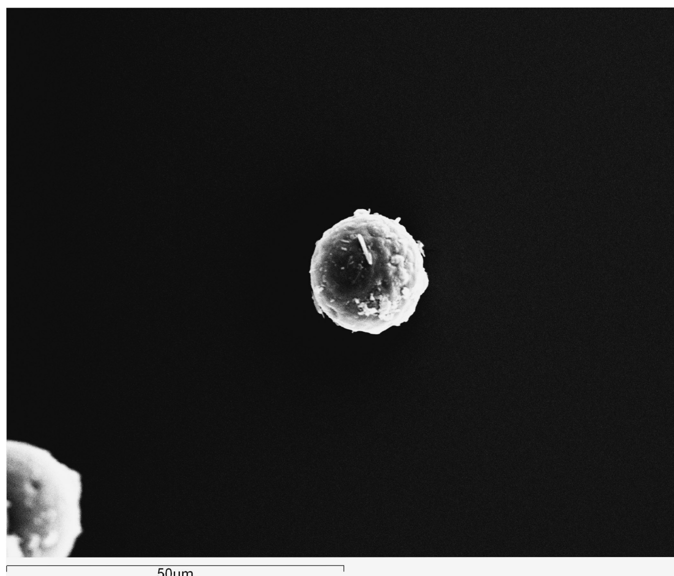

FIG. 1c. SEM micrograph of B_1.

in varying the albumin derivatization degree. Collectively, all these results suggest that solvent penetrates easily through the pores, cavities, and/or channel of microparticles created.

The fast swelling and the high value of water content percentage suggest good ability of prepared matrices to release drug molecules in a physiological medium. To estimate the ability of A/DMAA (A_1, A_2) and B/DMAA (B_1, B_2) matrices to release drug molecules, beads were loaded with various drugs. The incorporation of drugs during the crosslinking process has not been possible because of their inadequate solubility in aqueous dispersed phase.

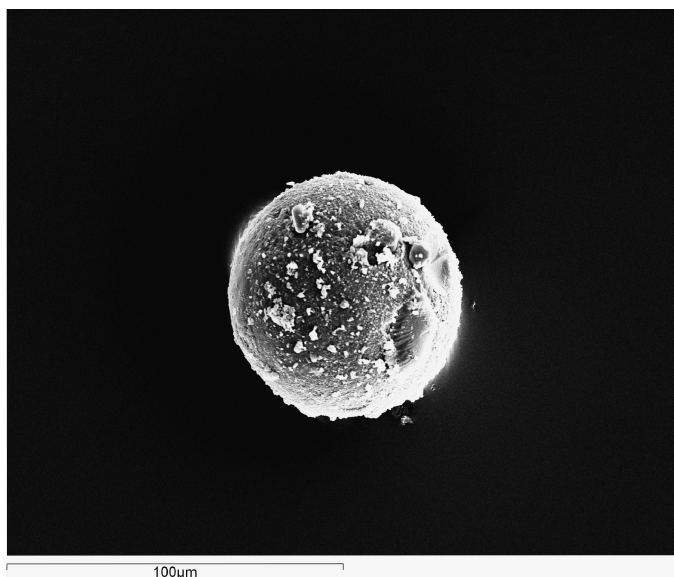

FIG. 1d. SEM micrograph of B_2.

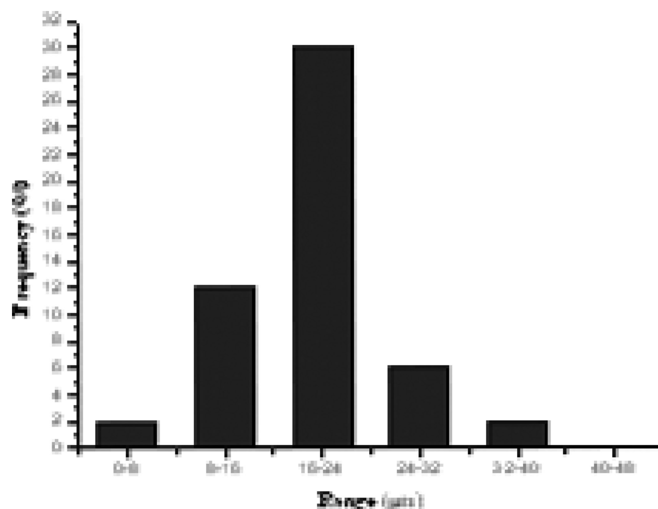

FIG. 2a. Size distribution profiles of A_1.

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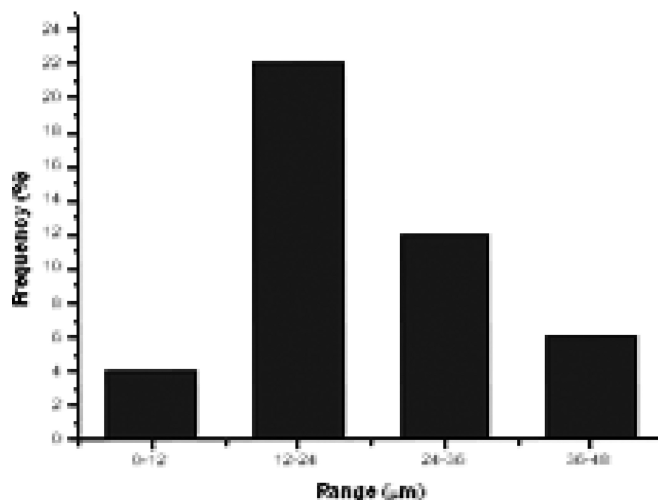

FIG. 2b. Size distribution profiles of A_2.

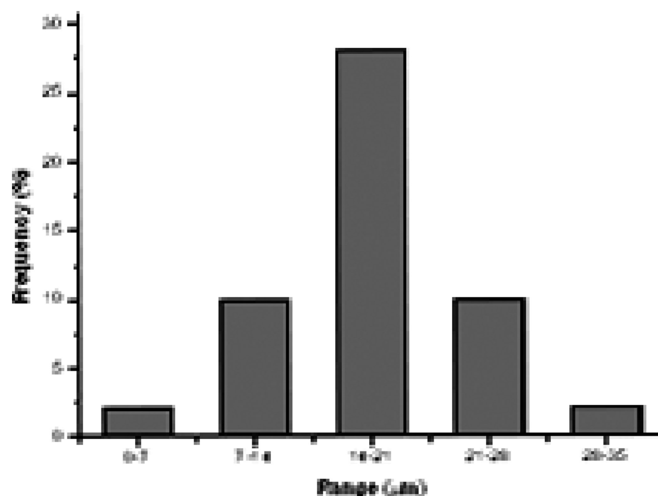

FIG. 2c. Size distribution profiles of B_1.

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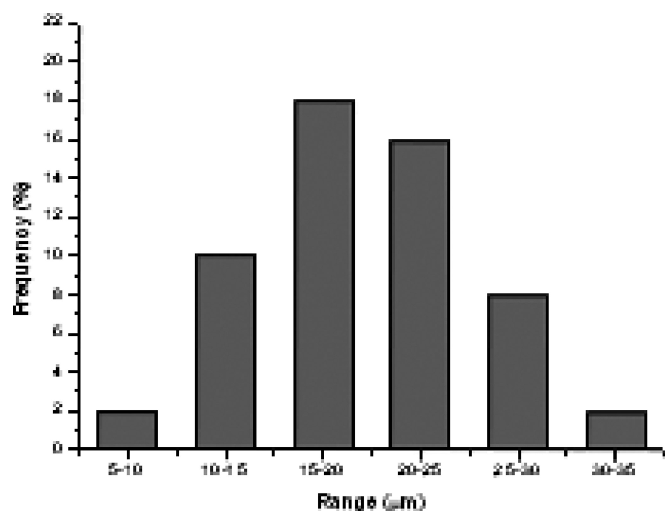

FIG. 2d. Size distribution profiles of B_2.

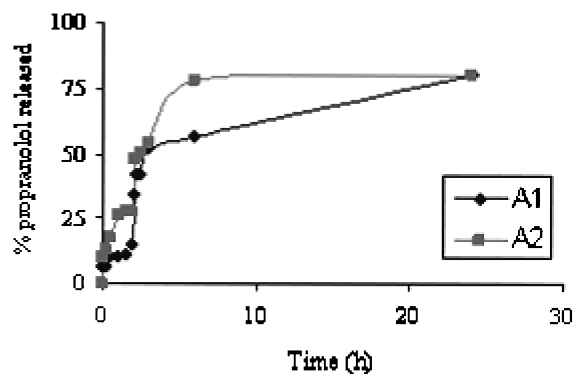

FIG. 4a. Release of β-propranolol at pH 1 from 0 to 2 hr and at pH 6.8 from 2 to 20 hr (sample A_1 and A_2).

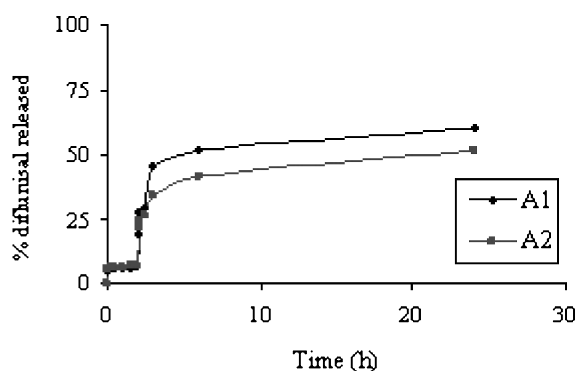

FIG. 3a. Release of diflunisal at pH 1 from 0 to 2 hr and at pH 6.8 from 2 to 20 hr (sample A_1 and A_2).

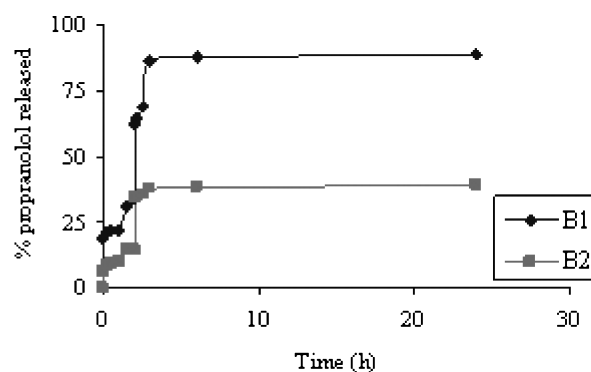

FIG. 4b. Release of β-propranolol at pH 1 from 0 to 2 hr and at pH 6.8 from 2 to 20 hr (sample B_1 and B_2).

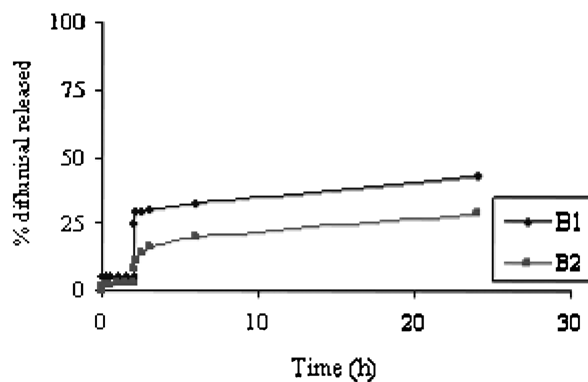

FIG. 3b. Release of diflunisal at pH 1 from 0 to 2 hr and at pH 6.8 from 2 to 20 hr (sample B_1 and B_2).

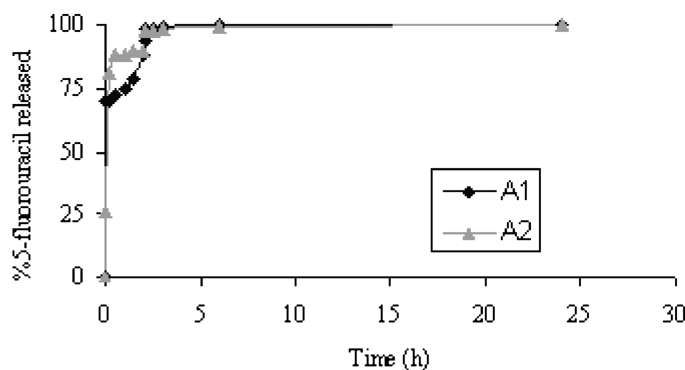

FIG. 5a. Release of 5-fluorouracil at pH 1 from 0 to 2 hr and at pH 6.8 from 2 to 20 hr (sample A_1 and A_2).

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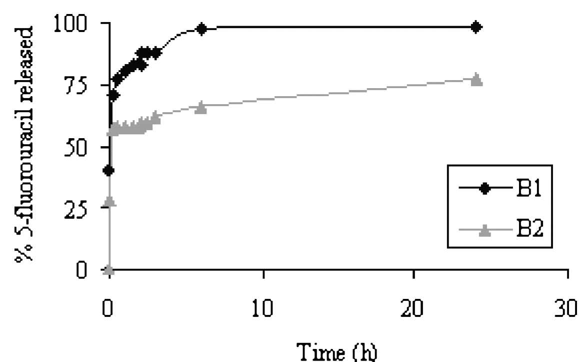

FIG. 5b. Release of 5-fluorouracil at pH 1 from 0 to 2 hr and at pH 6.8 from 2 to 20 hr (sample B_1 and B_2).

The drugs have been loaded on microparticles by soaking procedure after the crosslinking reaction. The experiments have been carried out at 37°C at pH 1 (simulated gastric fluid) and pH 6.8 (simulated intestinal fluid) using the pH change method (see Methods section). The drug release was expressed as the percent of drug (related to the entrapped total dose) delivered as a function of time from A_{1-2} and B_{1-2} matrices. Figures 3a–3b depict drug release of diflunisal from microspheres with two different crosslinking degrees.

The experimental data showed a limited release of diflunisal from beads also after pH change because of the known strong interaction that is established between albumin and drugs with acid properties. Thus an incomplete release was observed. This effect is more marked in beads with a greater content in albumin and with higher crosslinking degree.

For β-propranolol the release was greater regarding the diflunisal release (Figures 4a–4b). Moreover, it catches up approximately 80% for A_{1-2} and B_1 beads. On the contrary, the β-propranolol release from B_2 microparticles is minor. Finally, 5-fluorouracil is quickly released at pH 1 and becomes complete within 3 hr (Figures 5a–5b).

It is possible to observe a remarkable variation in the amount of drug released for B_2 beads. For diflunisal, the variation in the amount of drug released at pH 6.8 is caused by salification of acid drug and by a greater ability to swell to pH 6.8 of microparticles, although the release remains incomplete because of the strong interaction between the drug and albumin-based matrices. With a basic drug, like β-propranolol, the release profile shows a greater percentage at pH 1 within 2 hr, because in these conditions the ionized form of the drug prevails. However, it is interesting to observe the different profile release of drug in enviromental pH values in which these drugs are in undissociate form (diflunisal pH $<$ pKa; β-Propranololo pH $>$ PKb). A particular performance has been observed for B_2 particles loaded with β-propranolol. In this case about 40% of the drug is released at pH 6.8 and it remains incomplete within 24 hr. We probably can assume that great interactions are established between the β-propranolol and the polymeric network, thus reducing the drug release.

Finally for 5-fluorouracil that does not undergo salification in aqueous medium, no different release is noted for A_1, A_2, and B_1 varying the pH values. Only for B_2 was lower release observed and ascribable to swelling properties and crosslinking degree of this matrix.

CONCLUSIONS

BSA derivatized and DMAA were employed for preparing materials crosslinked by a reverse phase suspension polymerization method. All obtained microparticles showed a spherical shape, a porous surface, and a narrow size distribution. Swelling studies revealed a pH-dependent behavior in media that simulate gastrointestinal fluids. The applicability of these materials as drug delivery systems has been evaluated by loading drugs with different chemical properties by a soaking procedure. In particular, for this material, the drug release features depend principally on crosslinking degree, ratio among albumin and DMAA, and interactions of "loaded drug-beads." In fact, for diflunisal and β-propranolol the parameter most influential is the interaction with matrix, strong for the first and weak for the second, and less important the crosslinking degree and composition of matrix. For the system β-propranolol becomes important in the crosslinking degree. The 5-fluorouracil release decreases when the crosslinking degree is very high and it is poorly influenced by others parameters.

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