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Glutaraldehyde Cross-linked Bovine Casein Microspheres as a Matrix for the Controlled Release of Theophylline: In-vitro Studies

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Abstract—A controlled release dosage form of theophylline in the form of microspheres using the milk protein casein as the matrix is described. Glutaraldehyde cross-linking of an aqueous alkaline solution of the protein containing the drug, dispersed in a mixture of dichloromethane/hexane having ca. 1% of an aliphatic polyurethane as the suspension stabilizer, led to the formation of the drug-loaded microspheres. Drug incorporation efficiency of around 80% could be achieved by the technique. Release profiles of the drug were examined in simulated gastric and intestinal fluids at 37°C. It was observed that the release was diffusion-controlled and followed the Higuchi model. Release characteristics were influenced by the cross-linking density, particle size and the extent of loading. Data obtained indicate that the natural milk protein casein could be used as a matrix for sustained release oral dosage forms.

Theophylline is an effective drug for the treatment of acute and chronic bronchial asthma (Anderson et al 1983) and children suffering from the disease are often given this drug for treatment (Kern & Lipman 1977). Therapeutic efficacy will be achieved with minimal toxicity by maintaining the plasma concentration between 10 and 20 μg mL^{-1} (Ogilvie 1978). Even though there are several sustained release dosage forms of theophylline in the form of tablets, children are often unable to swallow them (Motycka et al 1985). Liquid preparations, on the other hand, have to be administered more frequently, as the absolute bioavailability of orally administered theophylline in aqueous solution is 100% and peak serum concentration is reached 1 h after administration (Hendeles et al 1977).

Motycka et al (1985) prepared a sustained-release formulation of theophylline in the form of a drug/ion-exchange resin complex and suggested a suspension of these beads in a suitable vehicle as a prolonged dosage form for paediatric use. Even though polymeric microspheres have attracted considerable attention as drug carriers (Davis et al 1984), few reports have dealt with the preparation and evaluation of theophylline-loaded polymeric microspheres as a sustained release dosage form (Benita & Donbrow 1982; Lin & Yang 1987; Pongpaibul et al 1988; Thanoo et al 1992). As originally suggested by Motycka et al (1985), microsphere-based formulations would be particularly suitable for paediatric use.

This report is concerned with the preparation and in-vitro evaluation of theophylline-loaded, glutaraldehyde-cross-linked casein microspheres as a sustained release oral dosage form.

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Materials and Methods

Materials

Bovine casein, glutaraldehyde (25% biological grade) and theophylline were purchased from Sigma Chemical Co., St Louis, USA, and were used as such. Biomedical grade aliphatic polyurethane Tecoflex 80A from Thermedics Inc., Woburn, USA, was employed as the suspension stabilizer. All solvents were of analytical grade. Simulated gastric and intestinal fluids without enzymes were prepared according to the US Pharmacopeia. Simulated gastric fluid was prepared by dissolving 2 g NaCl in 7·0 mL HCl and making up to 1000 mL with water (pH = 1·2). Simulated intestinal fluid was prepared by dissolving 6·8 g monobasic potassium phosphate in 250 mL water, adding 190 mL of 0·2 M NaOH and 400 mL water, adjusting the pH to 7·5 and making up to 1000 mL with water.

Preparation of microspheres

A 20% solution of casein was prepared in 0·5 M NaOH. The required amount of theophylline was mixed with 2 mL of the casein solution and suspended in a dispersion medium consisting of 40 mL dichloromethane and 25 mL hexane containing 1% (w/v) polyurethane as the steric stabilizer in a 100 mL round-bottomed flask. The polyurethane solution was prepared in dichloromethane by dissolving the polymer overnight and then diluting with hexane. The dispersion was stirred at 1000 rev min^{-1} using a stainless steel half-moon paddle stirrer and 5–10 mL glutaraldehyde-saturated toluene (Longo et al 1982) was added depending on the cross-linking density desired. Stirring was continued for 1 h at room temperature (27°C). After decanting the solvent, the microspheres were washed with a 1 : 1 mixture of dichloromethane/hexane and centrifuged at 2000 rev min^{-1}. Washing and centrifugation were repeated 5–6 times to remove all the polymeric stabilizer. They were then washed once with

acetone followed by 1% sodium bisulphite, 0·1 M HCl, distilled water and finally with acetone. Microspheres thus obtained were desiccated.

Characterization of microspheres

Particle size distribution. Particle size distribution of the microspheres was determined by sieving 500 mg of the microspheres through standard test sieves (Filterwel, Bombay, India) and weighing the fractions that passed through each sieve. The percentage weight fraction was then plotted against the particle size. Deviations, if any, in the particle size distribution were further checked using microscopy. Fifty to sixty spheres from randomly selected fractions were subjected to particle size measurement using an optical microscope. No deviation from the distribution was detected by this method, thereby confirming the reliability of the sieving method.

Microscopy. Scanning electron microscopy was performed using a Jeol instrument (Model JSM 35C). Microspheres were sprinkled onto double-sided tape, sputter-coated with gold and examined in the microscope.

Thermal studies. Differential thermal analysis (DTA) of the placebo spheres and the spheres loaded with theophylline was carried out using a Dupont (Model 990) thermal analyser system with a standard DTA cell accessory, in an atmosphere of nitrogen at a heating rate of 10°C min^{-1}.

Drug content. Determination of the extent of loading of theophylline in microspheres was carried out as follows. The drug-loaded microspheres (5 mg) were powdered in an agate mortar and extracted with 5 mL methanol in a screw-capped test tube over 3 days by rotating the tubes in a haematology mixer (Fisher, Model 346, USA). After filtering through a 0·45 μm filter, a known volume was made up in a 100 mL standard flask and the absorbance was measured at 274 nm in a UV-vis spectrophotometer (Hitachi, Model 220, Japan). No other component was found to absorb at this wavelength. The efficacy of the method was checked by powdering equal amounts of theophylline and placebo casein spheres, extracting the drug as before and estimating the concentration of the drug in the methanol extract spectrophotometrically. The reproducibility of the method was found to be within ±2%. A similar extraction procedure was validated in our earlier work (Thanoo et al 1992). All assays were carried out in triplicate. The incorporation efficiency of theophylline in the microspheres was determined from the ratio of the weight of theophylline incorporated to the weight of theophylline initially taken. The percentage actual and theoretical loadings reported are calculated on the basis of the amount of theophylline incorporated and the amount of theophylline initially employed for 100 g casein.

Kinetics of swelling in simulated biofluids. The kinetics of swelling of placebo casein spheres in both simulated biofluids were examined in the following manner. A sample of microspheres, 0·1 g, was taken in different test tubes and 10 mL of the biofluids was added into each tube and incubated at 37°C. Tubes were taken at different time intervals, the spheres were filtered and blotted using a Whatman No. 1 filter paper and transferred into small beakers and weighed. Microspheres were then washed with acetone and dried to constant weight at 60°C in an air oven. From the difference in weight of the dry and swollen spheres, the fluid content was estimated using the following relation:

$$\text{Fluid content (\%)} = 100 \times \frac{\text{Wt of swollen spheres} - \text{Wt of dry spheres}}{\text{Wt of swollen spheres}}$$

In-vitro release studies. Release studies were carried out in simulated gastric and intestinal fluids according to the USP procedure using the paddle-type dissolution apparatus. Drug-loaded microspheres (50 mg) were taken in 500 mL of the dissolution medium at 37°C and stirred at a speed of 120 rev min^{-1} using a Teflon paddle stirrer. Aliquots (3 mL) were removed at various time intervals and assayed spectrophotometrically as before. An equal volume of the dissolution medium was immediately added to maintain a constant volume. Each point on the release curves represents the average of at least three determinations with a standard deviation less than 5%.

Results and Discussion

Cross-linked casein microspheres have attracted attention recently as carriers for cytotoxic drugs (Chen et al 1987; Jayakrishnan et al 1993; Knepp et al 1993). It is also reported that removal of the free amino groups in the protein reduces its proteolytic susceptibility (Samanen 1985). Aldehyde cross-linking of casein would result in the removal of most of the free amino groups in the protein and this is expected to impart stability to the microspheres in the gastrointestinal tract, as we have demonstrated in this work. Unlike synthetic polymers which are employed for the preparation of drug-loaded microspheres, biopolymers have the advantage of better biocompatibility and non-toxicity. This is particularly true with the milk protein casein as it forms an integral part of our daily diet.

The amphiphilic protein casein is insoluble in water, but can be solubilized at alkaline pH. A 20% solution of casein in 0·5 M NaOH is homogeneous and can be dispersed in a non-aqueous dispersion medium as good spherical droplets using a suitable stabilizing agent. Although low hydrophilic lipophilic balance-surfactants such as sorbitan sesquioleate were capable of producing a good dispersion of casein in non-aqueous solvents at a concentration of around 2% (Jayakrishnan et al 1993; Knepp et al 1993), it was found that the aliphatic polyurethane Tecoflex 80A provided steric stabilization of the protein solution droplets at much lower concentrations (ca. 1%). Goldberg et al (1984, 1987) and Longo et al (1982) have described several advantages of using polymers as steric stabilizers for the preparation of albumin spheres which included improved hydrophilicity, better particle size control and the absence of adsorbed surfactants that might influence tissue reactions and drug release. However, they used a 25–30% solution of polymers such as poly(methyl methacrylate) for the preparation of albumin spheres. The method reported here uses a 1% solution of the polymer to obtain microspheres of good spherical geometry and strength. Microspheres containing more than 50% theophylline could be prepared by this

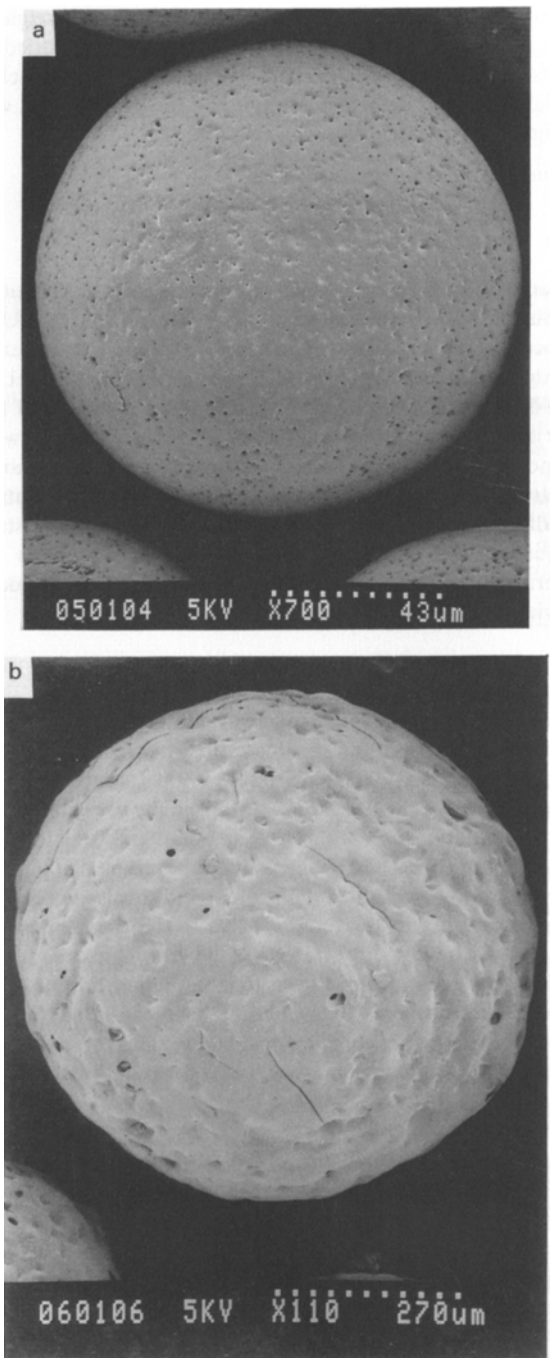

FIG. 1. Scanning electron micrograph of placebo casein spheres (a) and casein spheres containing 54% theophylline (b).

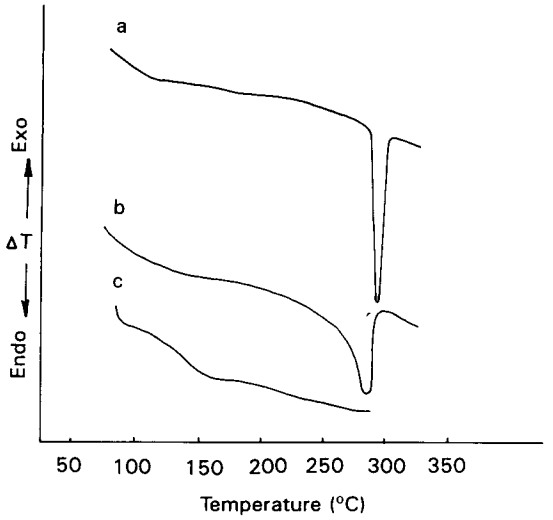

FIG. 2. DTA profiles of theophylline (a), casein spheres loaded with 54% theophylline (b) and placebo casein spheres (c).

method. Fig. 1 shows the scanning electron micrograph of placebo casein spheres and spheres loaded with 54% theophylline. The proteolytic stability of the spheres was checked using pepsin, papain and α-chymotrypsin. Simulated gastric fluid containing 0·32% pepsin (USP standard) at 37°C was not found to digest the microspheres even after 24 h. Microscopic examination of the spheres before and after incubation did not show any degradation. Addition of trichloroacetic acid into the incubation medium did not show any sign of protein precipitation. Also papain and α-chymotrypsin at concentrations of 0·5 and 0·2% in phosphate buffer did not show any degradation of the spheres at 37°C for 24 h. Thus, it can be presumed that glutaraldehyde cross-linked bovine casein spheres would not be susceptible to enzymatic attack during its transit in the gastrointestinal tract.

The DTA plots of the free drug, the placebo microspheres and the spheres loaded with 54% theophylline are shown in Fig. 2. The endotherm of the free drug at 275°C shows the melting of the drug crystals. In the drug-loaded spheres, this endotherm persists, although shifted very slightly to a lower temperature, proving that the drug remains as a crystalline dispersion inside the microspheres. This small shift in the endotherm can be attributed to a difference in the particle size of the free drug and the drug in the protein matrix. There are several reports in the literature that the ΔT_{min} in the DTA curves is prone to shift on change in particle size (Wendlandt 1974); the slight shift seen in the present study is therefore believed to be insignificant. The physical state of the drug inside the matrix is reported to be important as it affects the stability of the drug and its release rate (Benoit 1985). The particle size distribution of casein microspheres with different drug loadings is shown in Fig. 3. The weight fraction of particles having a diameter less than 300 μm is less than 5% in all preparations at different drug loadings when all the other variables were kept constant. With increase in drug content, the fraction having larger particles is increased which could be attributed to a viscosity effect. As expected, the incorporation efficiency of theophylline is increased with increasing concentration of the drug in the dispersed phase (Table 1). As there is no chemical reaction between the matrix polymer and theophylline, the loading is achieved by simple physical entrapment of the drug.

The release rates of theophylline from casein microspheres in simulated gastric and intestinal fluids are shown in Fig. 4. Microspheres of 710–850 μm were employed in the study. The solubility of theophylline in distilled water at 25°C is 1 g in 120 mL (Swinyard 1980). The maximum amount of the drug released in the experiments described would be 33 mg in 500 mL, approaching a solubility of around 0·8% thereby

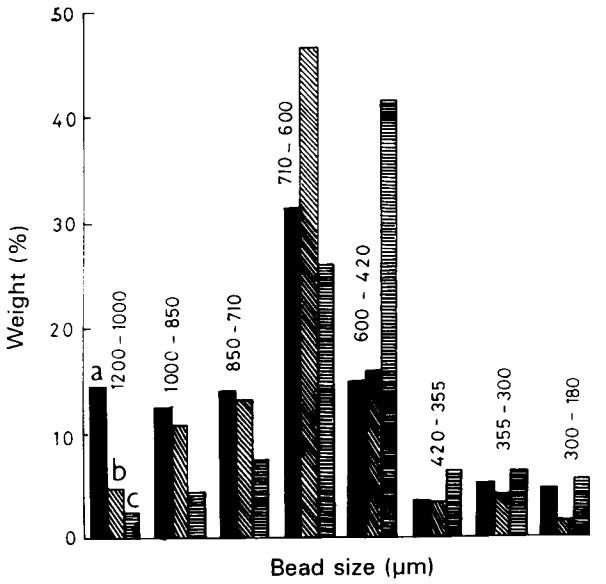

FIG. 3. Particle size distribution of casein microspheres with different theophylline loadings prepared using 1% polyurethane as the stabilizer at a stirring speed of 1000 rev min^{-1}. a 54%, b 45% and c 32%.

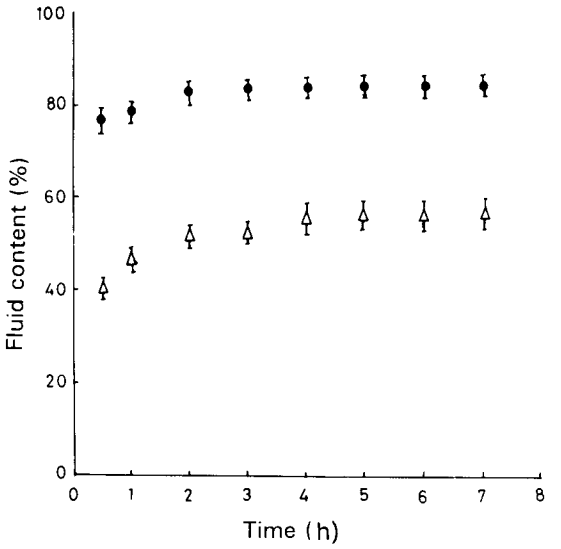

FIG. 5. Rate of swelling of placebo casein spheres cross-linked using 10 mL glutaraldehyde in simulated gastric fluid (△) and in simulated intestinal fluid (●).

maintaining sink conditions in all experiments. The rate of release in gastric fluid is slower than in intestinal fluid. This is in accordance with the expectations because the matrix swells more at pH 7·4 because of the slow conversion of the protein into its sodium salt form, thereby opening the pores more to facilitate more rapid diffusion of the drug. While 100% of the drug is released in about 5 h in intestinal fluid, it takes nearly 8 h for complete release to be achieved in gastric fluid. Determination of the swelling rates of the placebo spheres in gastric and intestinal fluid showed that though the equilibrium swelling is attained in both fluids in the same fashion, microspheres swelled to a greater extent in intestinal fluid compared with gastric fluid as expected (Fig. 5). The greater swelling seen in intestinal fluid is believed to be due to the slow ionization of the protein into its sodium salt form. In fact, when microspheres prepared from the alkaline solution of casein were acidified using HCl after the bisulphite wash, considerable shrinkage in particle size could be observed. The average decrease in diameter of the spheres on acidification was determined for 25 spheres before and after acidification using an optical microscope and was found to be $10 \pm 3\%$. Thus, the drug in acidified casein spheres is more tightly packed than in the sodium salt of casein. Further evidence of this effect was obtained when the rate of release of theophylline was examined from casein microspheres in their sodium salt form into gastric and intestinal fluids (Fig. 4). Compared with the release from acidified casein spheres, the release was faster from spheres in the sodium salt form in both gastric and intestinal fluids.

Table 1. Incorporation efficiency of theophylline in casein microspheres at different initial drug loadings.

Theoretical loading (%)	Actual loading (%)	Incorporation efficiency (%)
66	54	82
60	45	75
50	32	64

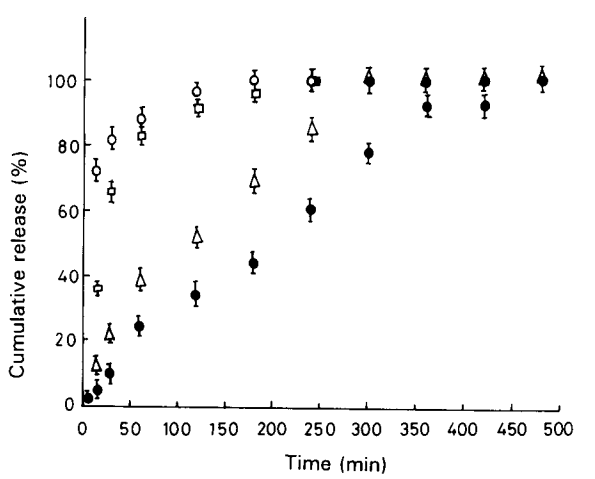

FIG. 4. In-vitro release profiles of theophylline from casein microspheres of 710–850 μm cross-linked using 10 mL glutaraldehyde containing 54% drug, into simulated gastric and intestinal fluids at 37°C. ● Gastric fluid from spheres in the acid form, △ intestinal fluid from spheres in the acid form, □ gastric fluid from spheres in the sodium salt form, ○ intestinal fluid from spheres in the sodium salt form.

The rate of release is also very much dependent on the cross-linking density of the spheres. Although the glutaraldehyde concentration employed for cross-linking the spheres was very small as it was introduced via an organic medium as a saturated solution in toluene, the effect of cross-linking density on the release rates was found to be remarkable (Fig. 6). The introduction of the cross-linking agent via the organic phase is believed to cross-link the spheres extensively on the surface with less cross-linking inside the matrix, thereby providing a surface net controlling the diffusion of the drug from the matrix. While it takes 7 h for complete release of the drug from spheres cross-linked with 10 mL glutaraldehyde-saturated toluene, it took only about 2 h for

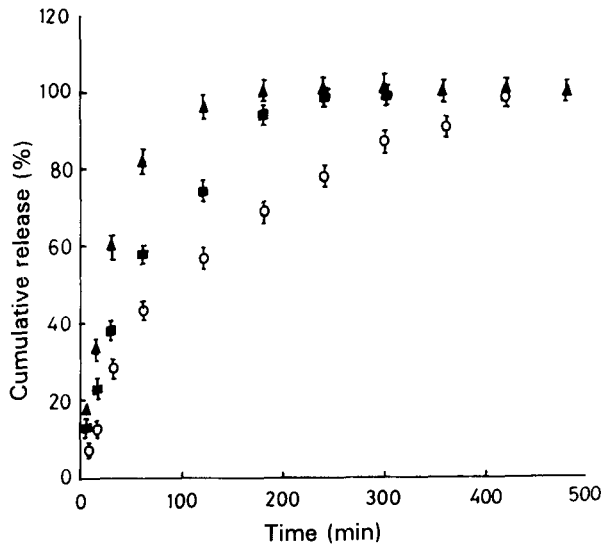

FIG. 6. Effect of cross-linking density on the release profiles of theophylline from casein microspheres of 425–350 μm size containing 32% theophylline at 37°C. ▲ Cross-linked with 6, ■ with 8, ○ with 10 mL glutaraldehyde-saturated toluene.

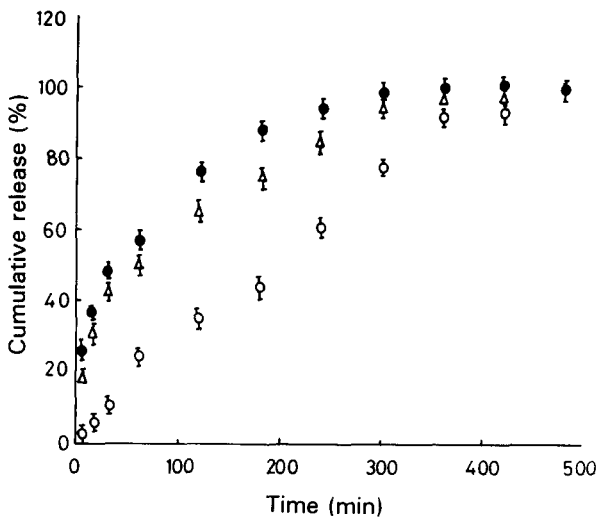

FIG. 7. Rate of release of theophylline from casein microspheres containing 54% theophylline cross-linked with 10 mL glutaraldehyde-saturated toluene as a function of microsphere size at 37°C. ○ 850–710 μm, △ 600–425 μm, and ● 425–350 μm.

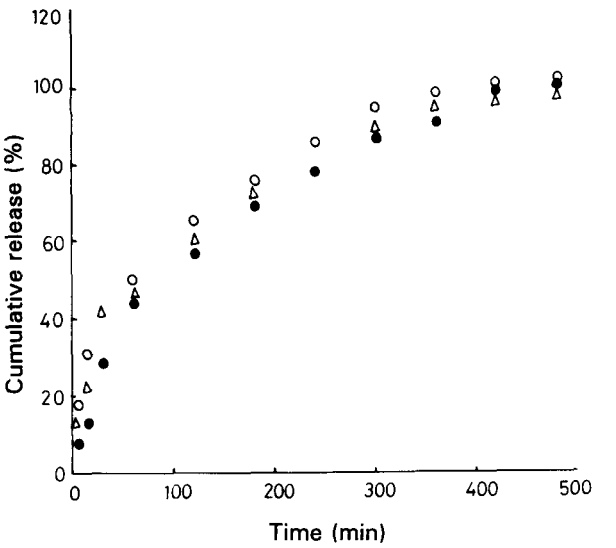

FIG. 8. Rate of release of theophylline from casein microspheres of 600–425 μm cross-linked using 10 mL glutaraldehyde-saturated toluene as a function of drug loading at 37°C. ○ 54%-, △ 45%- and ● 32%-loaded spheres.

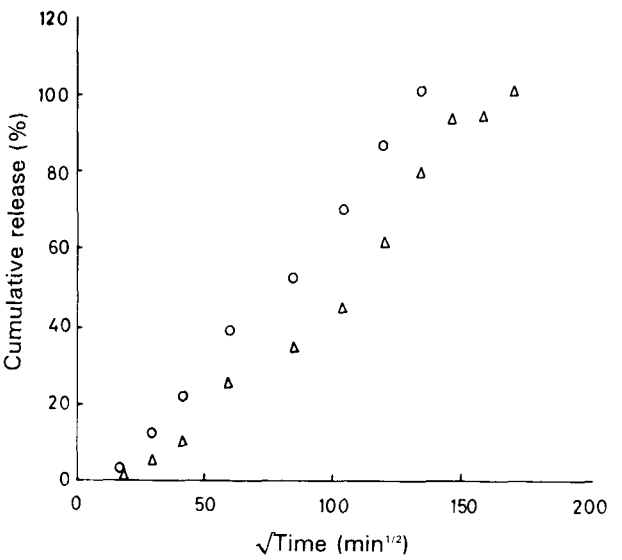

FIG. 9. Plots of theophylline released vs square root of time in simulated gastric (△) and intestinal fluid (○) at 37°C from microspheres loaded with 54% theophylline having particle size 710–850 μm.

complete release from spheres cross-linked with 6 mL glutaraldehyde. Thus, it is possible to modulate the release of drugs from the protein matrix by changing the surface cross-linking density.

The rate of release was also influenced by the microsphere size to a significant extent. Data illustrated in Fig. 7 show that release rate from smaller microspheres was faster than the rate from larger spheres in accordance with the expectation that smaller spheres by virtue of larger area of contact with the dissolution medium would release the drug at a faster rate. Modulation of the release rate would thus be possible by mixing spheres of various sizes.

The extent of loading also affects the release rate from the spheres (Fig. 8). Increase in loading increases the release rate. With decrease in loading, the fraction of the matrix polymer that hinders the diffusion of the drug is increased thereby reducing the rate of release. At high loadings, however, this effect is not very pronounced as can be seen in the case of 54%- and 45%-loaded spheres.

The release of drug from a matrix-type device where the release is diffusion controlled is given by the Higuchi model (Higuchi 1963),

$$Q = kt^{1/2} \quad (1)$$

where Q is the amount released in time t and k depends on the surface area and the diffusion coefficient. Assuming that surface area and diffusion coefficient to be constant throughout the experiment, a plot of Q vs $t^{1/2}$ should be linear (Fig. 9).

Both plots gave a good fit with correlation coefficients 0·998 and 0·997 for simulated gastric and intestinal fluids, respectively.

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