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Casein as a Carrier Matrix for 5-Fluorouracil: Drug Release from Microspheres, Drug-protein Conjugates and In-vivo Degradation of Microspheres in Rat Muscle

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Abstract—Glutaraldehyde cross-linked casein microspheres were loaded with 5-fluorouracil (5-FU) from concentrated aqueous solutions of the drug after the microspheres were synthesized and cleaned. In-vitro release of the drug was examined in phosphate buffer in the absence and in the presence of protease at 37°C. Drug release data showed that only about 20% of the drug is released in the absence of protease even after 5 days, while digestion of the matrix with protease released the entrapped drug completely in about 24 h. A protein-drug conjugate was synthesized via carbamoyl linkage using 6-(5-FU-1-yl)hexyl isocyanate and the drug release was examined in phosphate buffer at 37°C. Release from the protein-5-FU conjugate was slower compared with the release from microspheres in the presence of protease. Implantation of placebo microspheres of different cross-linking densities in the gluteal muscle of rats showed no adverse tissue reactions over a one-year period. Histopathological examination of the tissues containing injected microspheres suggested that the biological life of casein microspheres in muscle is about 6 months, which is three times that of cross-linked albumin microspheres.

5-Fluorouracil (5-FU) is an antitumour drug used in the treatment of carcinoma of the colon, rectum, breast, stomach and pancreas (Heidelberger 1982; Waxman & Scanlon 1982). However, severe side-effects have been cited for this drug (Bosch et al 1958; Bounous et al 1978). To counter the side-effects, various attempts have been made to control the tissue distribution and pharmacokinetics of the drug. Approaches made in this direction include synthesis of monomeric prodrugs having clinical activity with reduced toxicity (Kundu & Schmitz 1982), polymeric prodrugs fixing 5-FU onto the backbone of natural as well as synthetic polymers (Ouchi et al 1989, 1990) and microencapsulation of the drug in protein, polysaccharide and other polymeric matrices (Sugibayashi et al 1977; Ghorab et al 1990; Ohya et al 1993).

The approach of incorporating the drugs within a microsphere matrix either in the native form or by attachment to the matrix is reported to reduce the plasma concentration of the drug and is better suited to target the drug to the desired site (Goldberg et al 1987; Willmott 1987). Kerr et al (1988) have shown that administration of doxorubicin in microspherical form reduced the plasma concentration within 1 h of administration by 85–90% compared with the drug in solution while maintaining the concentration in target organs. Of the various protein and polysaccharide microspheres investigated for controlled drug delivery, microspheres based on the serum protein albumin have attracted considerable attention (Morimoto & Fujimoto 1983; Gupta & Hung 1989; Arshady 1990). Recently, carriers based on the milk protein casein in the microsphere form have been investigated and appear to be more promising than albumin. Chen et al (1987) compared the therapeutic efficacy of doxorubicin containing casein and albumin microspheres for mammary carcinoma in Wistar rats and found that casein spheres containing the drug were more effective than albumin. Willmott et al (1989, 1992) investigated the biodegradation rate of embolized casein and albumin spheres containing doxorubicin in lung, liver and kidney of rats using radiolabelled particles and found that the casein spheres degraded slowly compared with those from albumin. We have recently shown that glutaraldehyde cross-linked casein microspheres could be used as a carrier for orally administered drugs such as theophylline, and that proteolytic enzymes at concentrations prevailing in the gastrointestinal tract do not degrade the spheres during its transit (Latha & Jayakrishnan 1994). We have also shown that casein microspheres incorporating a relatively new anticancer drug mitoxantrone is more effective against Lewis lung carcinoma compared with the free drug when administered intratumorally in mice (Knepp et al 1993). Casein, therefore, appears to be a promising carrier for the sustained release of many oral as well as parenterally administered drugs. Being a more hydrophilic matrix in its sodium salt form compared with albumin, casein spheres offer the significant advantage of incorporating high payloads of water-soluble drugs from their aqueous solutions after the microspheres are synthesized. This is particularly useful for drugs such as methotrexate, adrenaline and salbutamol whose activities are affected by aldehyde cross-linking (Gupta & Hung 1989) or for drugs such as adriamycin which have low thermal stability (Gupta et al 1987). Furthermore, such post-synthesis loading has the unique advantage of removing all the impurities and solvent residues from the microspheres before the drugs are loaded in their native form.

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This study was undertaken with a view to incorporating an antineoplastic agent such as 5-FU in its native form as free drug into preformed casein spheres and to examine the release of the entrapped drug from the microsphere matrix, to synthesize a protein-drug conjugate in an unambiguous manner, to examine the release of the covalently bound drug from the matrix, and to examine the biological life of the casein microspheres intramuscularly implanted over a period of one year to assess the biodegradability and the toxicity of the microsphere preparations.

Materials and Methods

Materials

Bovine casein, glutaraldehyde (25% biological grade), trichloroacetic acid, protease (Type VIII), hexamethylene diisocyanate, pyridine, glycine, and 5-FU were purchased from Sigma Chemical Co., St Louis, USA, and were used as received. Tecoflex 80 A, a biomedical grade aliphatic polyurethane employed as the suspension stabilizer was from Thermedics Inc., Woburn, USA. All other reagents and solvents were of analytical or equivalent grade.

Preparation of microspheres

Casein microspheres were prepared as reported earlier (Latha & Jayakrishnan 1994). Briefly, 2 mL 20% casein in 0·5 M NaOH was dispersed in a mixture of 40 mL dichloromethane and 25 mL hexane containing 1% polyurethane as the suspension stabilizer and stirred at 1500 rev min^{-1} using a paddle stirrer. Cross-linking of the spheres was carried out by adding 10 mL glutaraldehyde-saturated toluene prepared according to the method of Longo et al (1982) and stirring the dispersion for 1 h. Microspheres were washed using dichloromethane/hexane several times followed by acetone, water and acetone, and dried. Quenching of the residual glutaraldehyde or the aldehyde moieties present on the spheres was carried out using glycine. Microspheres were equilibrated in a 20% solution of glycine in water, centrifuged, washed several times with water followed by acetone and dried. Microspheres were sieved using standard test sieves (Filterwel, Bombay, India). Particles of 75–150 μm range were used for all experiments.

Incorporation of 5-FU

Aqueous solutions of 5-FU (1·5 and 3·0%) were made in distilled water by heating to ca 60°C. Approximately 100 mg of the spheres was equilibrated in 2 mL of the drug solution at 60°C for 6 h. Spheres were centrifuged, washed once with distilled water and dried in vacuum. Glycine-quenched microspheres were also loaded with 5-FU in a similar fashion from a 3% solution. Drug incorporation is expressed as weight in g of 5-FU incorporated into 100 g of microspheres (wt %).

Determination of drug content

Drug-loaded microspheres were digested using protease and analysed for the total drug content. Microspheres (2 mg) were added to 10 mL phosphate buffer containing 2 mg protease and incubated at 37°C. After complete digestion, the protein was precipitated using trichloroacetic acid and centrifuged. The aqueous layer was analysed for 5-FU using a UV-vis spectrophotometer (Shimadzu, Japan) at 266 nm. The accuracy of the method was ascertained by incubating a known amount of the drug and placebo spheres in protease solution, precipitating the protein after digestion using trichloroacetic acid and analysing drug as before. Protein digestion and precipitation did not interefere with the determination of the drug content.

Equilibrium fluid uptake by microspheres

Microspheres were equilibrated in distilled water, phosphate buffer (0·1 M, pH 7·4) and phosphate-buffered saline (PBS) at 27°C and the equilibrium uptake of the fluids was determined from their dry weight and swollen weight as reported previously (Latha & Jayakrishnan 1994).

Preparation of 5-FU-casein conjugate

The 6-(5-fluorouracil-1-yl)hexyl isocyanate was prepared according to the method of Ouchi et al (1989). To 0·15 g 5-FU in a 50-mL round-bottomed flask was added 10 mL dry pyridine and the mixture was stirred with a magnetic pellet. After complete dissolution, 0·25 g hexamethylene diisocyanate was added and the contents stirred for 2 h at 90°C. To this solution 0·5 g casein was introduced and stirring continued for 24 h at room temperature (27°C). Precipitation of the conjugate was effected by the addition of acetone. The precipitate was washed with pyridine, followed by copious amounts of water, dried and ground in an agate mortar to particle size less than 200 μm.

Determination of 5-FU content in conjugate

Drug conjugate (6 mg) was refluxed with 10 mL 3 M NaOH for two days in a 50-mL round-bottomed flask. Two millilitres of this solution was mixed with 2 mL 3 M HCl and the precipitated protein removed by centrifugation at 2000 rev min^{-1}. The clear solution was analysed for 5-FU at 266 nm spectrophotometrically.

In-vitro drug release

Drug release from microspheres was followed in phosphate buffer. Microspheres (50 mg) were added to 100 mL buffer (0·1 M, pH 7·4) at 37°C and stirred at 100 rev min^{-1} using a Teflon paddle stirrer. Aliquots of 1·5 mL were withdrawn at intervals and 5-FU content was analysed as before. An equal volume of buffer was added to the medium after withdrawal of each aliquot. Drug release from microspheres was also followed in the presence of proteolytic enzymes. In this case, the protein in the aliquot was precipitated using trichloroacetic acid and centrifuged. The supernatant was filtered through a 0·45 μm filter (Sigma, USA) and then analysed for the drug content. All estimations were carried out in triplicate. Drug release from the protein-5-FU conjugate was followed in the presence of protease in a similar fashion.

Biodegradation of microspheres

The in-vivo degradation of placebo casein spheres injected intramuscularly into rats was studied over a one year period. Wistar rats were employed in the study. Microspheres (5 mg) were suspended in 1 mL physiological saline and injected into the gluteal muscle using a 21 G needle. Each animal received two injections on either side of the muscle.

Animals were killed, and the tissue plus the microspheres at the site of injection were removed and fixed in 10% buffered formalin. Sections having a thickness of 5 μm were cut, stained with haematoxylin and eosin and were examined in the microscope for the extent of biodegradation and tissue compatibility.

Results and Discussion

Microspheres prepared as described were found to be extremely hydrophilic and completely non-aggregatory when suspended in water, phosphate buffer or saline. Since casein is an amphiphilic protein, insoluble in water, a solution of the protein could be made in alkali or alkaline buffers wherein the protein exists in its alkali metal salt form. In phosphate buffer, casein did not give rise to a homogeneous solution at a concentration of 10–20%, whereas in 0·5 M NaOH, a fairly homogeneous solution could be prepared which could be readily dispersed as microdroplets in a suitable organic dispersion medium. Thus, the spheres prepared are in the sodium salt form of casein. Acidification with HCl reduced the swelling characteristics of the spheres considerably (Table 1). A high payload of water-soluble drugs could therefore be incorporated in the microspheres, taking advantage of their high swelling characteristics. The amount of 5-FU incorporated into quenched and unquenched casein spheres from drug solutions of two different concentrations is shown in Table 2.

Interestingly, there is no difference in the drug uptake of quenched and unquenched spheres and the drug uptake is a function of the concentration of the drug initially present in solution. The unquenched spheres would presumably have residual unreacted aldehyde moieties on the matrix, but in the case of 5-FU this obviously has not made any difference in the drug uptake because of the absence of free amino groups (as opposed to, for example, adriamycin) that can participate in the formation of a Schiff's base linkage with the matrix.

The in-vitro release profiles of the drug from unquenched casein spheres are shown in Fig. 1. Only 10–20% of the incorporated drug is released even after five days. The release from glycine-quenched microspheres was virtually the same as the release from unquenched microspheres containing similar drug payloads (data not shown). A higher rate of release is seen from spheres having higher loading as expected (Fig. 2). The fact that only 20% of the incorporated drug is released over the time period studied demonstrates that most of the drug incorporated is bound to the protein matrix in some way. Similar behaviour has been observed for various drugs incorporated into protein microspheres such as albumin. Chen et al (1988) have reported that adriamycin incorporated into the protein matrix is covalently bound to it to some extent and the bound drug is available only on degradation of the matrix. Fujimoto et al (1983) have observed that the amount of mitomycin C released over a period of five days from albumin microspheres is only ca 20%. We have recently reported that a relatively new antitumour drug, mitoxantrone incorporated into albumin as well as casein microspheres is released only to the extent of 15% in-vitro over 24 h and the release rate reached a constant rate thereafter (Knepp et al 1993). Sugibayashi et al (1979) found that the release of 5-FU from heat-denatured albumin microspheres was only 10–30% in about 24 h and thereafter remained constant for several days. The covalent attachment of drugs having amino functions onto the carrier matrix during glutaraldehyde cross-linking is a distinct possibility which, however, cannot be ascribed to drugs such as 5-FU.

By the post-loading technique employed, one would imagine that the drug is incorporated into the spheres simply by physical entrapment, but the mechanism of incorporation seems to be very complex. Previous workers in this area have also reported that the mechanism of entrapment and release of drugs from protein matrices appears to be complex and is not fully understood (see, for example, the review by Morimoto & Fujimoto (1983)). However, no attempt seems to have been made to elucidate

Table 1. Equilibrium fluid content (%) of placebo casein microspheres in their sodium salt form and in the acid form in various fluids at 27°C.

Fluid	Casein-Na^+	Casein-H^+
Distilled water	95 ± 2·2	24 ± 2·9
Phosphate buffer 0·1 M	83 ± 2·9	61 ± 1·6
Phosphate-buffered saline	76 ± 3·3	49 ± 4·1

Mean ± s.d. (n = 3)

Table 2. Amount of 5-FU incorporated into glycine-quenched and unquenched casein microspheres (75–150 μm) from drug solutions of two different concentrations at 60°C.

Drug concn (wt %)	Amount incorporated (wt %)
1·5	4·4 ± 0·15 (Quenched)
3·0	7·1 ± 0·05 (Quenched)
3·0	7·7 ± 0·4 (Unquenched)

Mean ± s.d. (n = 3).

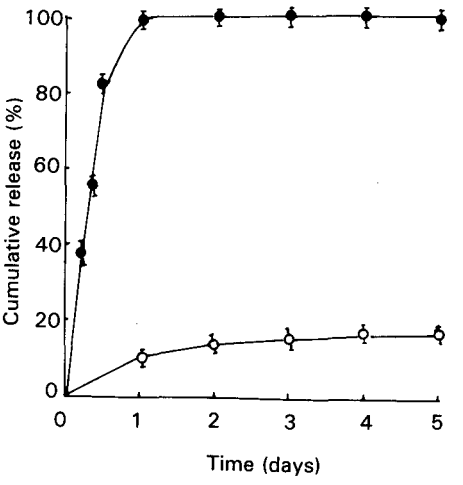

FIG. 1. In-vitro release of 5-FU from unquenched casein microspheres (75–150 μm) cross-linked using 10 mL glutaraldehyde-saturated toluene having a 5-FU content of 7% in the absence (○) and in the presence (●) of 0·005% protease at 37°C.

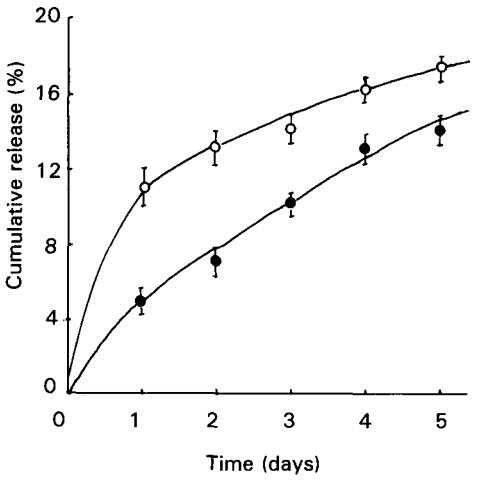

FIG. 2. In-vitro release of 5-FU from casein microspheres (75–150 μm) cross-linked using 10 mL glutaraldehyde-saturated toluene having 4·1% 5-FU (●) and 7·1% 5-FU (○).

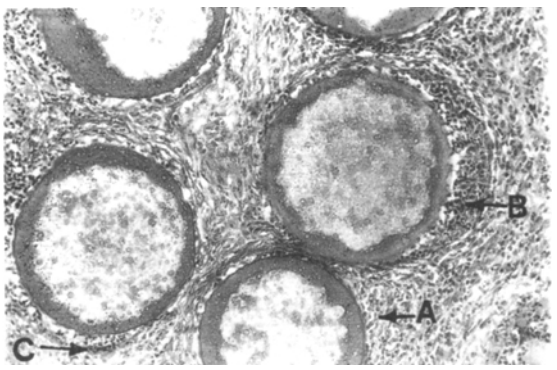

FIG. 4. Histological section of rat skeletal muscle containing casein microspheres cross-linked with 15 mL glutaraldehyde-saturated toluene in their sodium salt form 7 days post-implantation. The spheres are homogeneous and circular with numerous fibroblasts and macrophages (A) in between. Neutrophils (B) invade the sphere material and an occasional foreign body-type giant cell is also seen (C).

the mechanism of entrapment. From the data obtained, it appears to us that the drug molecules are firmly held by the entangled polypeptide chains with no covalent attachment. The complete release of 5-FU from the microsphere matrix seen on protease digestion supports this view (Fig. 1).

Polymer-drug conjugates are believed to have a prolonged duration of activity. 5-FU was attached to the casein matrix using hexamethylene diisocyanate. Analysis of the drug content of the conjugate showed that the loading is ca 15%. As opposed to the drug-loaded microspheres, the conjugate was more resistant to protease digestion. At comparable protease concentrations (0·005%), while 100% of the drug was released from the microspheres loaded with 5-FU in its native form in 24 h, only about 10% is released from the protein-5-FU conjugate. Even at very high protease concentrations (0·05%), the release from the conjugate was only ca 20% in 24 h. Even prolonged incubation for five days did not release the drug completely (Fig. 3). This observation again suggests that the drug entrapment in the microsphere matrix is purely physical in nature.

Microspheres cross-linked with 15 and 30 mL glutaraldehyde-saturated toluene in their sodium salt form (Type I & II) and those cross-linked with 30 mL glutaraldehyde-saturated toluene followed by 3 mL 25% aqueous glutaraldehyde (Type III) were used for the in-vivo degradation studies. At seven days post-implantation, microspheres of all types were present in the skeletal muscle, with most of them clustered together (Fig. 4). The spheres had a homogeneous eosinophilic appearance. The inflammatory cellular infiltrate between spheres and around the implanted group as a whole consisted predominantly of fibroblasts and macrophages. Fibrocytes formed a single or double layer close to the microsphere margin. Infiltrate of neutrophils was found to invade the sphere material. Foreign body giant cells were seen adjacent to microspheres.

At six weeks post-implantation, intact microspheres were still seen and the neutrophil infiltration was absent. At six months post-implantation, broken degraded masses of microspheres were seen for all three types (Fig. 5). Microspheres still elicited a response of macrophages and

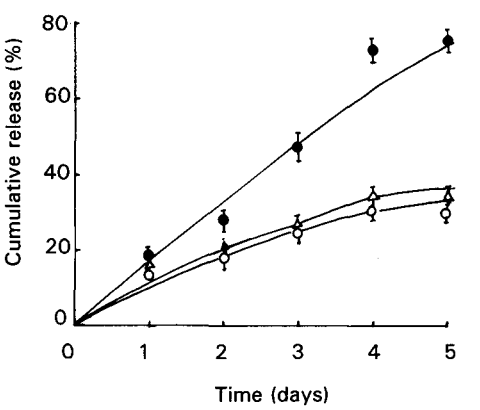

FIG. 3. In-vitro release of 5-FU from casein-5-FU conjugate having a 5-FU content of 15% in the absence (○) and in the presence of 0·005% (△), or 0·05% protease (●).

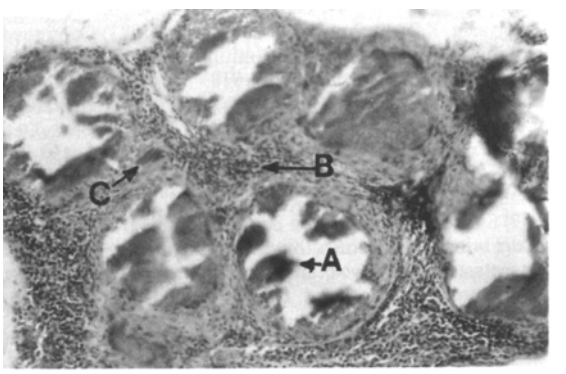

FIG. 5. Histological section of rat skeletal muscle containing fragments of casein microspheres (A) cross-linked with 15 mL glutaraldehyde-saturated toluene in their sodium salt form six months post-implantation demonstrating almost complete degradation of the microspheres. Cellular infiltrate of macrophages, lymphocytes and fibroblasts (B) persists, but to a lesser degree in comparison with a 7-day or a 6-week explant with occasional foreign body-type giant cells (C).

fibroblasts. Foreign body-type giant cells were still present adjacent to the spheres.

At 12 months post-implantation, it was very difficult to locate remnants of microspheres by gross examination. Microscopic examination revealed only brown globular masses at the implantation site with a few fibroblasts, fibrocytes and macrophages.

The histological picture indicated that the microspheres are well tolerated by living tissue with no adverse tissue reactions and the in-vivo life of the microspheres in the muscle is about six months. It has been reported that glutaraldehyde cross-linked albumin microspheres degrade completely in the rabbit muscle in two months (Lee et al 1981). Casein, therefore, appears to have an in-vivo life three times that of albumin. Although spheres of three different cross-linking densities were implanted, it was very difficult to discern the differences in the biodegradability of the spheres. Type III spheres, which are highly cross-linked, were expected to last longer in the tissue, but there appeared to be little evidence to this effect.

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