International Journal of Pharmaceutics, 95 (1993) 85–91

IJP 03075

Preparation, characterisation and in vitro stability of hydrophilic gelatin microspheres using a gelatin-methotrexate conjugate

R. Narayani and K. Panduranga Rao

Biomaterials Laboratory, Central Leather Research Institute, Adyar, Madras 600 020 (India)

(Received 13 August 1992)
(Accepted 5 October 1992)

Key words: Gelatin; Methotrexate; Microsphere; Conjugate; Release; Particle size

Summary

The anticancer drug methotrexate (MTX) was covalently linked to gelatin using carbodiimide as the coupling agent. The resulting gelatin-methotrexate conjugate (GMC) was separated by gel filtration and characterised by UV and IR spectroscopy. The drug content of the conjugate was 200 μg MTX/mg gelatin. The GMC was used to prepare biodegradable hydrophilic gelatin microspheres (GMCM) of different mean particle sizes (1–5, 5–10 and 15–20 μm). The in vitro release of MTX from GMCM was investigated in simulated gastric and intestinal fluids. GMCM released MTX in a zero-order manner for 7–9 days in gastric medium and for 8–10 days in intestinal medium. The release data also indicated that the rate of release of MTX decreased with increase in particle size of GMCM. Release of MTX was faster in gastric medium when compared to intestinal medium.

Introduction

The use of microspheres as controlled release targeting agents for anticancer drugs has received wide attention during recent years. (Ghorab et al., 1990; Nishioka et al., 1990; Ohya et al., 1991; Yan et al., 1991). Even though many biodegradable materials are used for the preparation of microspheres as carriers in cancer chemotherapy there are many limitations such as high temperature, use of surfactants and solvent. A simple and elegant method for the preparation of gelatin microspheres and their use for the controlled release of anticancer drugs such as bleomycin, mitomycin and 5-fluorouracil has been reported by us (Jeyanthi and Panduranga Rao, 1987).

Methotrexate (MTX) is one of the most widely used drugs for the treatment of neoplastic diseases in humans. Many macromolecular carriers have been investigated as drug delivery systems for methotrexate. Shen and Ryser (1979) have shown an increased cellular uptake in MTX-resistant cells using polylysine-MTX conjugate. Chu and Howell (1981) reported that the half-life of MTX can be increased when linked to bovine serum albumin. Kim and Oho (1988) reported the preparation of microspheres using an MTX-albumin conjugate. Immunoglobulin-MTX conjugate was prepared by Ghosh et al. (1988) to

Correspondence to: K. Panduranga Rao, Head, Biomaterials laboratory, Central Leather Research Institute, Adyar, Madras 600 020, India.

target tumour associated antigens. It was also reported that MTX-immunoconjugate synthesised by Shish and Goldenberg (1990) caused greater inhibition of tumour growth than free MTX.

The primary objective of this investigation was to synthesise a gelatin-MTX conjugate (GMC) and subsequently prepare biodegradable, hydrophilic gelatin microspheres containing the conjugated drug. The pH release profiles of MTX from the gelatin-MTX conjugate microspheres (GMCM) was studied in two different media, namely, simulated gastric fluid and intestinal fluid.

Materials and Methods

Materials

Methotrexate (MTX) was a gift sample from Tamil Nadu Dadha Pharmaceuticals Ltd, India. Gelatin (Oxoid, U.K.) and glutaraldehyde (25%) (Fluka) were used as obtained. Methyl methacrylate (MMA) (Sisco) was purified by distillation under reduced pressure. Potassium persulfate ($K_2S_2O_8$) and sodium bisulphite ($NaHSO_3$) were purchased from Loba, India. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDAC) was procured from Sigma. All other chemicals used were of analytical grade.

Methods

Synthesis of gelatin-MTX conjugate (GMC)

100 mg of gelatin was dissolved in 5 ml water with slight warming (40°C), cooled and adjusted to pH 4.7 with 0.01 N HCl. 25 mg MTX and 150 mg EDAC were added to the gelatin solution. The reaction was allowed to proceed at room temperature for about 6 h.

Purification of GMC

Gel chromatography The resultant conjugate solution was loaded on a Sephadex G-50 coloumn (17×2 cm i.d.) and equilibrated with phosphate buffer (pH 7.4). The coloumn was eluted with phosphate buffer pH 7.4 and 3 ml fractions of the eluant were collected (1 ml/min) and absorbance was read at 371 nm using a Shimadzu 2100-S UV/Vis spectrophotometer. The fractions containing the GMC were pooled and lyophilised for further characterisation.

Dialysis The conjugate was also purified by an alternative method. The crude conjugate was transferred into a dialysis bag and suspended in 500 ml phosphate buffer pH 7.4. The external buffer was frequently replaced with fresh buffer and periodically sampled and assayed at 371 nm spectrophotometrically to determine the amount of free MTX. The dialysis was continued for 60 h until there was no more release of free MTX from the crude conjugate.

Characterisation of GMC

Ultraviolet spectroscopy The purified GMC, gelatin and MTX were dissolved in phosphate buffer, pH 7.4 and scanned UV spectrophotometrically using the Shimadzu 2100-S UV/Vis spectrophotometer.

Fourier transform infrared spectroscopy The infrared spectra of GMC, gelatin and MTX were obtained using potassium bromide pellets in a Nicolet Model 20 DXB. Fourier transform infrared spectrophotometer.

MTX content of GMC The MTX content of GMC was determined from gel chromatography and dialysis experiments.

Preparation of microspheres using GMC Polymethylmethacrylate (PMMA) was prepared in our laboratory using distilled MMA by the $K_2S_2O_8$-$NaHSO_3$ redox initiation technique in aqueous medium as reported earlier (Jeyanthi and Panduranga Rao, 1987). A solution of GMC in phosphate buffer pH 7.4 was dispersed using concentrated PMMA solution in organic medium. The microspheres were crosslinked by addition of glutaraldehyde-saturated toluene. During the addition of PMMA and glutaraldehyde the gelatin solution was stirred using a vortex mixer. By varying the speed of mixing and concentration of PMMA and gelatin GMCM of three different sizes were obtained.

Characterisation of GMCM

Particle size analysis About 200 microspheres were randomly selected and their particle size

was measured using an optical microscope (Hertel Reuss, Germany) fitted with a micrometer scale. The particle size of GMCM was plotted against their percent frequency.

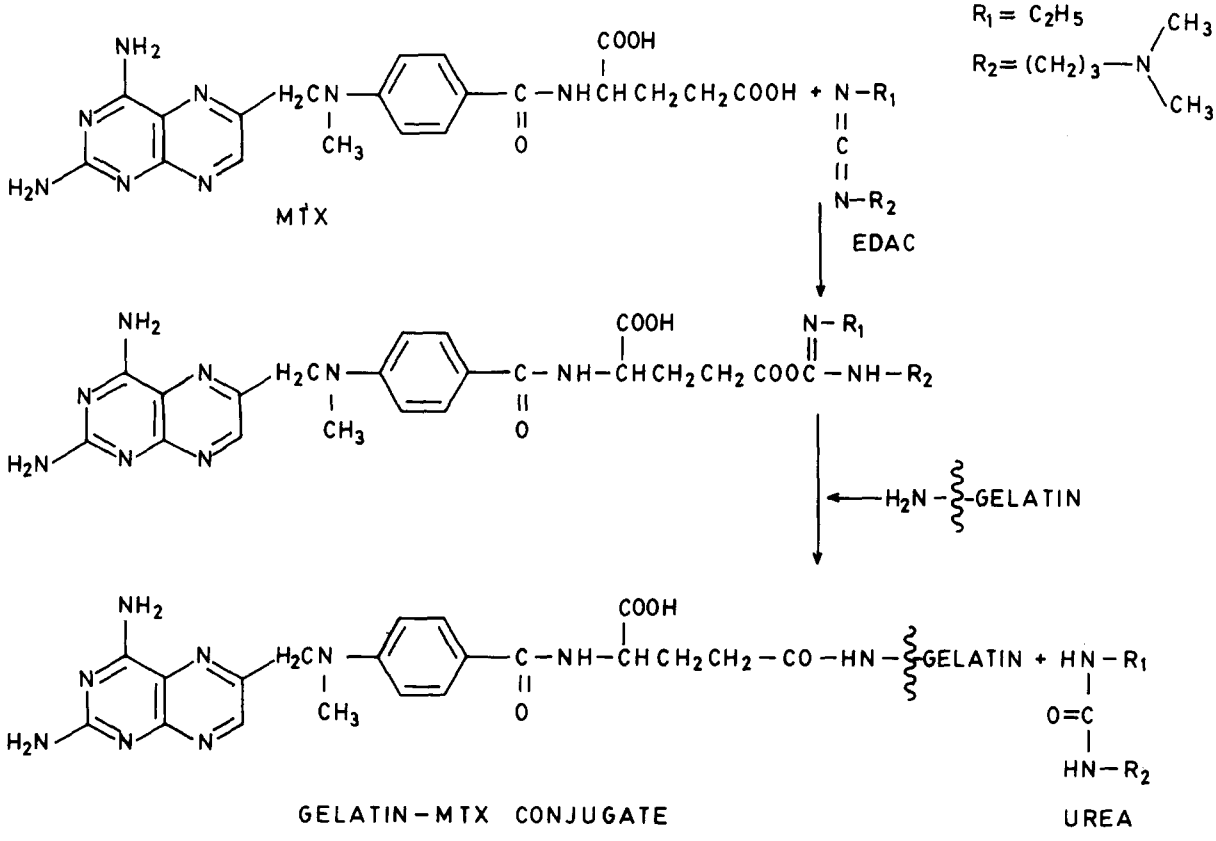
Optical microscopy and scanning electron microscopy The morphological characteristics of GMCM were studied using an optical and a scanning electron microscope (Cambridge Stereoscan S-150).

In vitro release studies of GMCM The release profiles of MTX from GMCM was determined in two different pH media. The experiments were carried out in simulated gastric fluid (0.1 N HCl, pH 1.2) and intestinal fluid (0.01 M phosphate buffer pH 7.4) using a Dissolution Test Apparatus (Veego Make VDA −6D) at 37°C and 100 rpm. The release medium was periodically sampled, filtered using a 0.45 μm Millipore filter and assayed spectrophotometrically at 243 and 371 nm for gastric and intestinal fluid, respectively.

Results and Discussion

Conjugation of MTX to gelatin

Carbodiimides have been used with great success in the synthesis of drug-macromolecular conjugates, to crosslink a carrier or drug to the amino group of the counterpart. In this investigation, MTX was conjugated to gelatin using EDAC as the coupling agent. Since EDAC is water soluble and active under mild aqueous conditions, it was considered suitable for bond formation between the carboxyl group of MTX and the amino group of gelatin. The mechanism of coupling is shown in Scheme 1. The GMC was characterised



Scheme 1. Mechanism of coupling of MTX to gelatin.

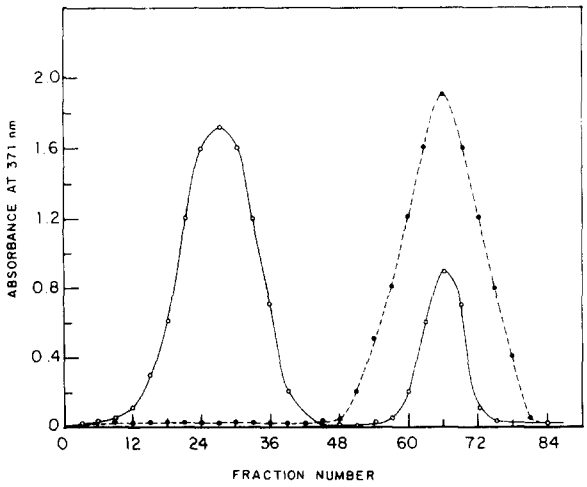


Fig. 1. Gel separation chromatogram of coupled MTX and free MTX on a Sephadex G-50 column eluted with 0.01 M phosphate buffer. (●- - - - -●) Gelatin + MTX, (○———○) gelatin + MTX + EDAC.

using drug elution patterns of the physical mixture of MTX and gelatin and GMC. It can be seen from Fig. 1 that from the physical mixture, the entire MTX content was detected as the small molecular fractions, whereas in the EDAC catalysed system, high absorbance of MTX was observed in both the macromolecular as well as the small molecular fractions. These results clearly indicated that the MTX detected in the macromolecular fraction pool existed as the conjugated form covalently bound to gelatin by EDAC.

Ultraviolet absorption of gelatin-MTX conjugate

Fig. 2A and B shows the UV absorption spectra of MTX and GMC. The UV spectra of MTX showed three absorption maxima, at 257, 302 and 372.5 nm. In the UV spectrum of GMC, the peaks at 257 and 302 nm were shifted to 261.5 and 309 nm, respectively, whereas that at 372.5 nm shifted to lower wavelength and showed a peak at 369.5 nm. Gelatin alone has no peak at these absorption maxima. The results clearly indicated the formation of a conjugate of MTX with gelatin.

Fourier transform infrared spectroscopy

The IR spectra were also used to establish that the conjugate was a chemical identity different from gelatin and MTX. The FT-IR spectra of GMC, gelatin and MTX are shown in Fig. 3. The infrared spectrum of gelatin showed the characteristic amide I and amide II peaks at 1651 and 1557 cm^{-1}, respectively. In the infrared spectrum of MTX, the aromatic ring stretching at 1575 cm^{-1} and the secondary amide peak at 1558 cm^{-1} can be seen. The IR spectrum of the gelatin-MTX conjugate showed the characteristic peaks which are present in the individual spectra of gelatin and MTX. A slight shift in some of the groups characteristic of MTX and gelatin took place due to conjugate formation. However, there is some overlapping of peaks, since both gelatin and MTX contain NH_2 groups. Further, the synthetic reaction conducted in the absence of EDAC did not yield a stable conjugate but only a physical mixture from which MTX could be rapidly dialysed out using phosphate buffer. This fact

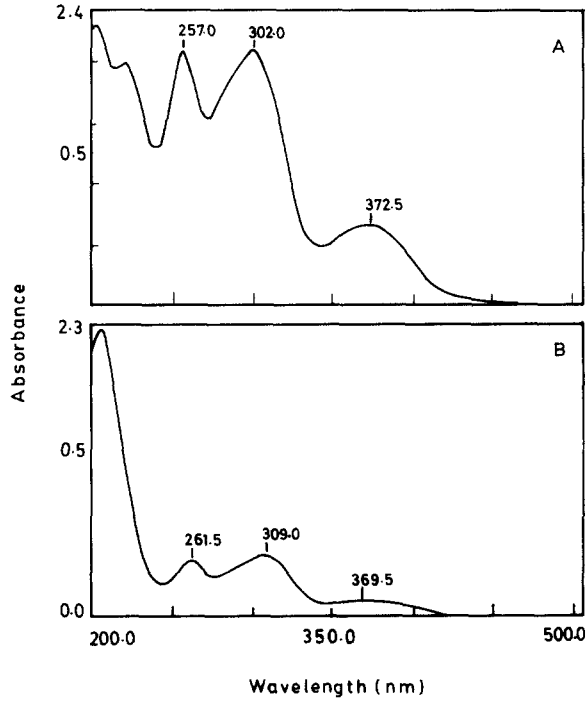


Fig. 2. Ultraviolet spectra of (A) MTX and (B) GMC in phosphate buffer pH 7.4.

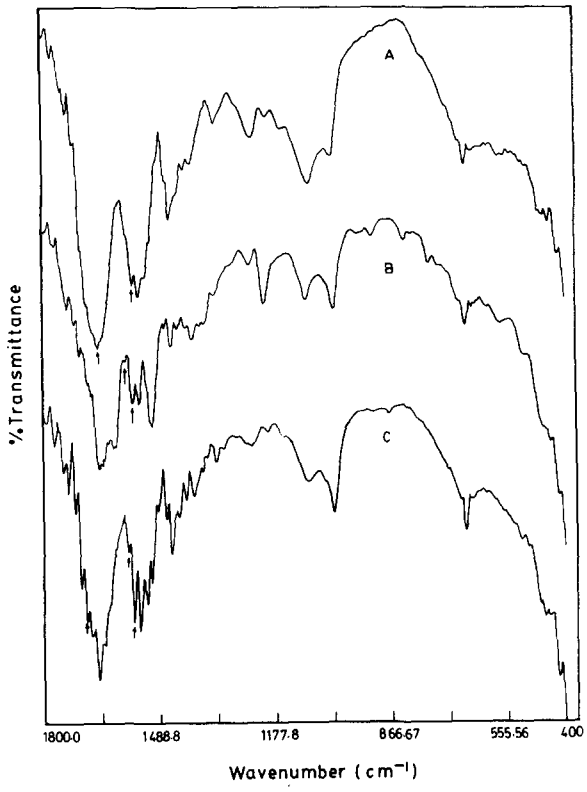


Fig. 3. Infrared spectra of MTX (A) gelatin, (B) MTX and (C) GMC.

along with the spectroscopic evidence established the formation of a covalent linkage between gelatin and MTX on reaction in the presence of EDAC.

MTX content of GMC

In the gel separation chromatogram of crude GMC one observes that two major peaks were eluted (Fig. 1). The first peak was due to GMC. The fractions which eluted as the second peak were pooled and the concentration of free MTX was determined. From the initial amount of MTX and that of free MTX, the amount of MTX coupled to gelatin was calculated. The amount of MTX coupled to gelatin was also determined using the dialysis method. This was evaluated from the difference between the total amount of MTX used in the conjugation and that which diffused out during dialysis. These values agreed well with those obtained by the gel chromatography method. 20 mg of MTX reacted with gelatin to form the conjugate with a drug content of 200 μg MTX/mg gelatin.

Particle size of GMCM

The size of GMCM was in the range of 1–20 μm. However, it was possible to obtain three types of microspheres by changing the experimental conditions such as stirring speed and concentration of PMMA and gelatin during the preparation of microspheres. As shown in Fig. 4, the mean particle sizes of three sets of GMCM were in the range of 1–5, 5–10 and 15–20 μm.

Optical and scanning electron microscopy

The optical photomicrograph (Fig. 5) and the scanning electron micrograph (Fig. 6) demonstrate the solid and smooth geometry of the microspheres.

In vitro release studies

Figs 7 and 8 show the in vitro release profiles of GMCM in simulated gastric and intestinal fluids, respectively. Fig. 7 demonstrates that GMCM of three different sizes released 96–98% MTX for 7–9 days in a zero-order fashion in gastric fluid. Fig. 8 shows that GMCM of different sizes released 94–96% MTX for 8–10 days in intestinal fluid. The release data also show that

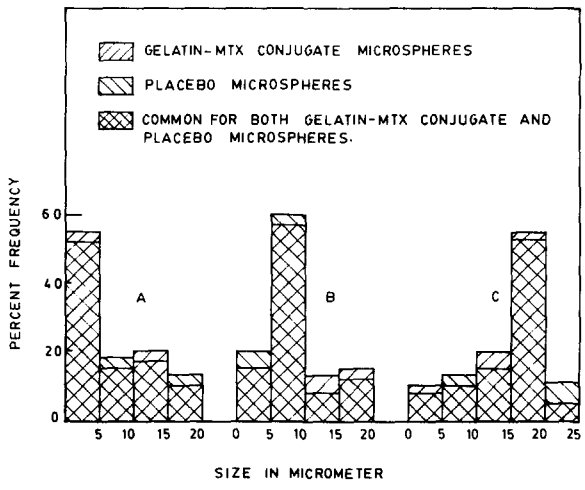


Fig. 4. Particle size distribution of gelatin-MTX conjugate microspheres. Mean particle size: (A) 1–5 μm, (B) 5–10 μm and (C) 15–20 μm.

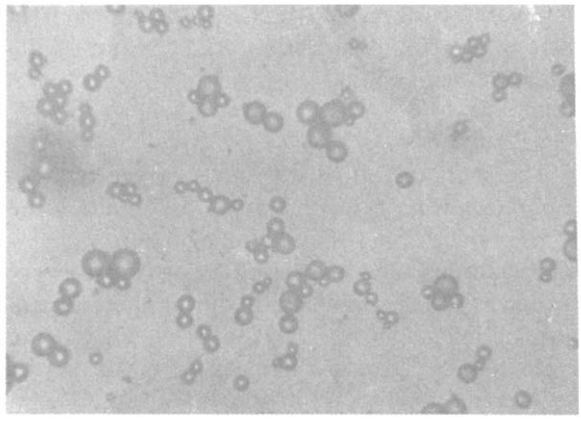


Fig. 5. Optical photomicrograph of gelatin-MTX conjugate microspheres. Mean particle size 5–10 μm.

the rate of release of MTX from GMCM was faster in gastric medium when compared to intestinal medium. From the figures, it is clearly seen that the rate of MTX decreases with increase in particle size of GMCM.

Effect of particle size of GMCM on MTX release

The rate of dissolution of drug from small size particles is higher due to their large surface area. Therefore, release of drug from smaller microspheres is faster when compared to larger microspheres (Figs 6 and 7). The in vitro release data of the present study showed that the rate of release of MTX from GMCM was in the following order: rate of release of MTX from GMCM : Microspheres 1–5 μm > microspheres 5–10 μm > microspheres 15–20 μm.

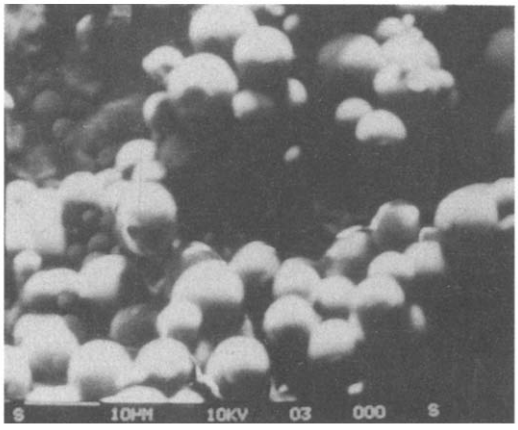


Fig. 6. Scanning electron micrograph of gelatin-MTX conjugate microspheres. Mean particle size 5–10 μm.

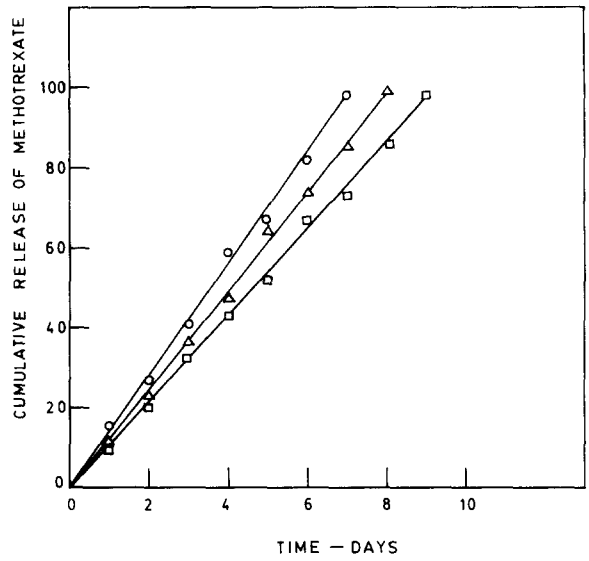


Fig. 7. In vitro release of MTX in 0.1 N HCl, pH 1.2 at 37°C from GMCM. Mean particle size: (○) 1–5 μm, (△) 5–10 μm and (□) 15–20 μm.

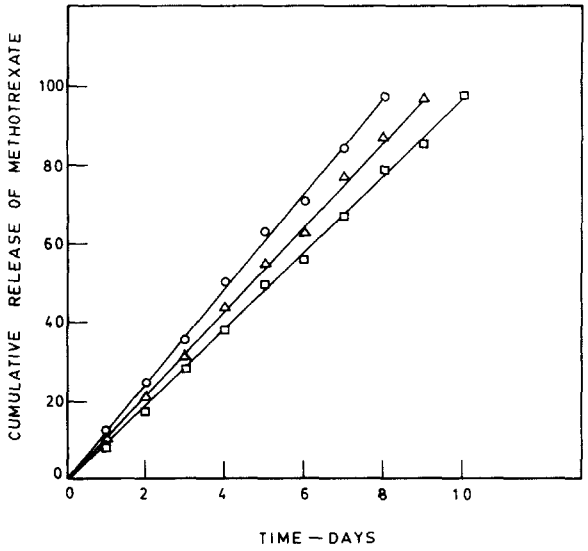


Fig. 8. In vitro release of MTX in 0.01 M phosphate buffer, pH 7.4 at 37°C from GMCM. Mean particle size: (○) 1–5 μm, (△) 5–10 μm and (□) 15–20 μm.

These results indicate that release rate of MTX increases with decrease in particle size of microspheres. Hence, it is possible to regulate the release of MTX for 7–10 days by varying the size of GMCM and the release medium.

Conclusion

The anticancer drug MTX was coupled to the natural and biodegradable macromolecular carrier gelatin by using carbodiimide as the coupling agent. Microspheres of different sizes prepared using the macromolecular conjugate released MTX in a controlled zero-order manner for prolonged periods in simulated gastric and intestinal fluids. This investigation shows that gelatin-MTX conjugate microspheres possess promising potential as a delivery system for MTX and thus help in avoiding the toxicity associated with the treatment using the free drug. An additional advantage of this system is that concomitant administration of gelatin–MTX conjugate microspheres and gelatin microspheres entrapping another free drug like mitomycin may be applied in the combination therapy of two synergistic drugs of schedule dependency. Moreover, a cocktail of two types of microspheres containing conjugated MTX and free MTX may be applied in cancer chemotherapy. In vivo studies in rat models are in progress and will be reported in a forthcoming article.

References

Chu, B.C.F. and Howell, S.B., Pharmacological and therapeutic properties of carrier bound methotrexate against tumor confined to a third space body compartment. *J. Pharmacol. Exp. Ther.*, 219 (1981) 389–393.

Ghorab, M.M., Zia, H. and Luzzi, L.A., Preparation of Controlled release anticancer agents: I.5-Fluororuracil-ethyl cellulose miscrospheres. *J. Microsencapsulation*, 7 (1990) 447–454.

Ghosh, M.K., Dane, K.O. and Mitra, A.K., Preparation and characterisation of methotrexate-immunoglobulin conjugates. *Drug Design Del.*, 4 (1988) 13–25.

Jeyanthi, R. and Panduranga Rao, K., Preparation of gelatin microspheres of bleomycin. *Int. J. Pharm.*, 35 (1987) 177–179.

Kim, C.K. and Oh, Y.K., Development of hydrophilic human serum albumin microspheres using a drug-albumin conjugate. *Int. J. Pharm.*, 47 (1988) 163–169.

Nishioka, Y., Kyotani, S., Okamura, M., Miyazaki, M., Okazaki, K., Ohnishi, S., Yamamoto, Y. and Ito, K., Release characteristics of cisplatin chitosan microspheres and effect of containing chitin. *Chem. Pharm. Bull.*, 38 (1990) 2871–2873.

Shen, W.C. and Ryser, H.J.P., Poly(L-lysine) and poly(D-lysine) conjugates of methotrexate: different inhibitory effect on drug resistant cells. *Mol. Pharmacol.*, 16 (1979) 614–622.

Shish, L.B. and Goldenberg, D.M., Effects of methotrexate-carcinoembryonic-antigen-antibody immunoconjugates on GW-39 human tumors in nude mice. *Cancer Immunol. Immunother.*, 31 (1990) 197–201.

Ohya, Y., Takei, T., Fuushima, H.F. and Ouchi, T., Preparation of albumin microspheres grafted galactoside residues through polyethylene-glycol spacers, release behaviour of 5-fluorouracil from them, and their *lectin* mediated aggregation. *J. Macromol.* Sci. Chem., 28 (1991) 743–760.

Yan, C., Li, X., Chen. X., Wang, D., Zhong, D., Tan, T. and Tano, H., Anticancer gelatin microspheres with multiple functions. *Biomaterials,* 12 (1991) 640–644.