ELSEVIER

International Journal of Pharmaceutics 128 (1996) 261–268

international journal of pharmaceutics

Biodegradable microspheres using two different gelatin drug conjugates for the controlled delivery of methotrexate

R. Narayani, K. Panduranga Rao*

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

Received 10 May 1995; revised 21 August 1995; accepted 8 September 1995

Abstract

The anticancer drug methotrexate (MTX) was covalently linked to gelatin by the azide coupling-grafting method. Two gelatin-MTX conjugates were prepared by coupling (i) MTX azide to gelatin (GMC-I) and gelatin azide to MTX (GMC-II). The resulting conjugates were separated by gel filtration and characterised by UV and IR spectroscopy. The drug content of GMC-I and GMC-II was 205 μg MTX/mg gelatin and 203 μg MTX/mg gelatin respectively. GMC-I and GMC-II were used to prepare biodegradable hydrophilic gelatin microspheres (GMCM-I and GMCM-II) of different mean particle sizes (1–5, 5–10, 15–20 μm). The in vitro release of MTX from GMCM-I and GMCM-II was investigated in simulated intestinal fluid. GMCM-I released MTX in zero order manner for 9–11 days in intestinal medium and GMCM-II released MTX in a zero order manner for 7–10 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.

Keywords: Biodegradable microspheres; Gelatin-drug conjugate; Methotrexate

1. Introduction

Researchers have often focused their attention on the attachment of low molecular weight drugs to macromolecular carriers. (Kotanagi et al., 1987; Takakura et al., 1987; Mukhopadhyay and Basu, 1990; Li et al., 1990; Hiroshi et al., 1992; Noguchi et al., 1992). Drugs can be covalently bound via spacers or directly to the chemical functional groups on the biodegradable polymer backbone. When a drug is bound to a polymeric carrier by linkage that is chemically or enzymatically cleaved in the biological environment such a conjugate can potentially maintain constant delivery of drug to the body over a given period. Poly(amino acids) like poly-L-lysine, poly (D-glutamic acid), antibodies, lectins, DNA and polysaccharides (Jeong and Kim, 1986) have been used to form drug-carrier conjugates. These polymer/macromolecular drug conjugates represent another new dimension in biodegradable polymeric drug delivery systems. The bioactive agent

* Corresponding author.

SSDI 0378-5173(95)04323-3

is liberated by the hydrolysis of the labile bond between the low molecular weight drug and the macromolecule which serves as the drug carrier.

The macromolecular drug carriers offer many potential advantages in drug delivery such as prolonged drug residence time of drugs in the compartment of application due to their very limited passage across compartmental barriers. Another reason for the prolongation of drug action is that their large size decreases the glomerular filtration rate and drug excretion. Moreover the conversion of low molecular weight drugs to a macromolecular form may restrict their distribution and limit damage to non-target tissues. The macromolecular carrier also protects the active moiety from inactivation e.g. by steric hindering of enzymatic attack and a similar steric factor renders the drug less immunogenic. Furthermore, the macromolecular carriers provide the opportunity of attaching specific ligands with tissue or organ affinity to control biodistribution of drugs.

It is well known that the adverse side effects of anticancer agents arise due to exposure of normal cells to therapeutically effective concentrations of the drug which is a major limiting factor for the use of cytotoxic drugs. Conjugates of the anticancer agents with polymers can be used to obtain more durable and moderate effect of the drug and prevent harmful secondary reactions (Jeong and Kim, 1986). It is reported by many researchers that macromolecular drug conjugates exhibited lesser toxicity and greater antitumor activity. Great uptake of drug by macrophages was observed when presented as conjugates of macromolecules and tumor cells exhibit a higher uptake of macromolecules by endocytosis than normal cells (Jeong and Kim, 1986). Therefore, conjugation of drugs to macromolecules provides a method for introducing substantial amounts of drugs to tumor cells and minimise systemic toxicity which would not be possible with the use of the pure drug only.

Methotrexate (MTX) is one of the most widely used drugs for the treatment of neoplastic diseases in humans. MTX-antibody conjugates have been extensively studied (Shen and Ryser, 1979; "Ghosh et al., 1988; "Shih and Goldenberg, 1990). Many macromolecular carriers have been investigated as drug delivery systems for methotrexate (Chu and Howell, 1981; Kim and Oh, 1988). We have earlier reported the preparation, characterisation and in vitro stability of hydrophilic gelatin microspheres containing gelatin-MTX conjugate using carbodiimide as coupling agent (Narayani and Panduranga Rao, 1993).

The aim of this investigation was to synthesise gelatin-MTX conjugates by the azide coupling-grafting method and subsequently utilise them for microsphere preparation. The functional groups of gelatin render it capable of covalent binding with a number of antitumor drugs while maintaining water solubility and thus serve as an ideal macromolecular drug carrier. The amino and carboxyl functional groups of both gelatin and MTX were utilised for the formation of conjugates. In this paper synthesis of gelatin-MTX conjugates, their characterisation and subsequent utilisation in the preparation of gelatin microspheres is given. The characterisation of the microspheres and the in vitro release studies of MTX from the microsphere is also reported.

2. Materials and methods

2.1. Materials

Methotrexate (MTX) was a gift sample from Tamil Nadu Dadha Pharmaceutical Limited, India. Gelatin (Oxoid, UK), glutaraldehyde (25%) (Fluka, Germany), Potassium persulfate and sodium bisulphite (Loba, India) were used as obtained. Methyl methacrylate (Sisco, India) was purified by distillation under reduced pressure. All other chemicals used were of analytical grade.

2.2. Synthesis of gelatin-MTX conjugates (GMC)

Two grams of gelatin and 500 mg of MTX were suspended each in separate conical flasks in excess of methanol made to 0.1 N with HCl at room temperature for 1 week for methylation. To the gelatin and MTX esters obtained was added 20 ml of 2% hydrazine hydrate each, at room temperature and left overnight. Each of the gelatin and

MTX hydrazides formed were treated with 80 ml of 0.6 N HCl with stirring at 0–5°C followed by the addition of 10 ml of 5% sodium nitrite dropwise. After 20 min the gelatin and MTX azides solutions obtained were adjusted to pH 9, using sodium hydroxide. A 10% solution of gelatin and 1% solution of MTX were added to MTX azide and gelatin azide solution respectively, at 0°C for 30 minutes. The resulting solutions were washed with toluene and acetone mixture and then with methanol to precipitate uncoupled MTX from MTX coupled to gelatin and dried well.

2.3. Purification of GMC

2.3.1. Gel chromatography

Each conjugate was loaded on a Sephadex G-50 column (17 × 2 cm i.d.) and eluted with phosphate buffer (PB), pH 7.4. The absorbance of 3 ml fractions collected (1 ml/min) were assayed spectrophotometrically at 371 mm. The fractions which eluted as the first peak containing GMC were pooled and lyophilised for further use.

2.4. Characterisation of GMC

2.4.1. Ultraviolet spectroscopy

The purified GMC-I, GMC-II, gelatin and MTX were dissolved in PB, pH 7.4 and scanned UV spectrophotometrically.

2.4.2. Fourier transform infra-red spectroscopy (FTIR)

The infra-red spectra of GMC-I, GMC-II, gelatin and MTX were obtained using KBr pellets in the Fourier transform infra-red spectrophotometer model Nicolet 20 DXB.

2.4.3. MTX content of the conjugates

To calculate the amount of MTX conjugated to gelatin, the amount of free MTX in the fractions which eluted as second peak during the purification of each GMC was determined spectrophotometrically.

2.5. Preparation of gelatin microspheres containing conjugated MTX (GMCM)

Microspheres were prepared by using polymethyl methacrylate (PMMA) in organic medium to disperse a solution of each of the conjugates in PB, pH 7.4 as reported earlier. Glutaraldehyde saturated toluene was used to crosslink the microspheres. By making appropriate changes in the concentration of PMMA, GMCM of three different size ranges were prepared in the case of each conjugate.

2.6. Characterisation of GMCM

The size of about 200 microspheres from each set was measured using a micrometer scale fitted to an optical microscope (Hertal, Reuss, Germany). The particle size was plotted versus % frequency for placebo microspheres and each set of GMCM.

2.7. In vitro release studies of GMCM

Known quantities of each set of GMCM were placed in a known volume of simulated intestinal fluid (0.01 M PB, pH 7.4) at 37°C and 100 rpm in a dissolution test apparatus (Veego Model VDA-6D, India). The MTX content of aliquots drawn periodically was assayed spectrophotometrically.

3. Results and discussion

3.1. Conjugation of MTX to gelatin and vice versa by the azide coupling method

Azides of MTX as well as gelatin were prepared and these azides were used for coupling to either gelatin or MTX. By this means two different types of conjugates were obtained. In the case of MTX, its carboxyl functionality was used in preparing its azide and then was coupled to the amino groups of gelatin (GMC-I). In the other case, the carboxyl groups of glutamic acid and aspartic acid of gelatin were used in the preparation of gelatin azide and then it was coupled to the amino functional group of MTX (GMC-II). Fig. 1a and Fig.

1b are the gel separation chromatogram of GMC-I and GMC-II respectively. It can be seen from Fig. 1a and Fig. 1b that from the physical mixture the entire amount of MTX was detected as the small molecular fractions, whereas in the case of conjugates synthesised by the azide coupling method the high optical density of MTX was observed at the macromolecular fractions as well as at the small molecular fractions. These results clearly indicated that the MTX detected at the macromolecular fraction pool existed as the conjugated form covalently bound to gelatin.

3.2. Characterisation of GMC-I and GMC-II

3.2.1. Ultraviolet absorption of gelatin - MTX conjugates

Fig. 2a, Fig. 2b and Fig. 2c shows the UV absorption spectra of MTX and GMC-I, GMC-II

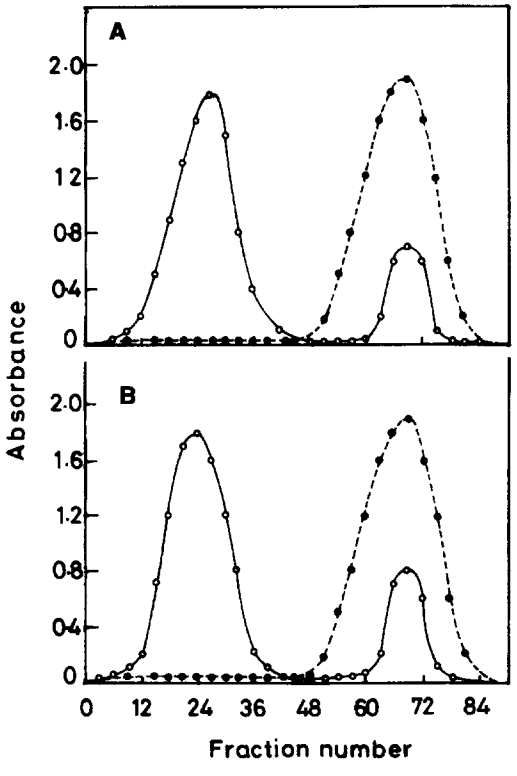

Fig. 1. Gel separation chromatogram of (a) GMC-I and (b) GMC-II on a Sephadex column G-50 eluted with 0.01 M PB -○-○ Gelatin + MTX; ○-○- Gelatin MTX conjugate.

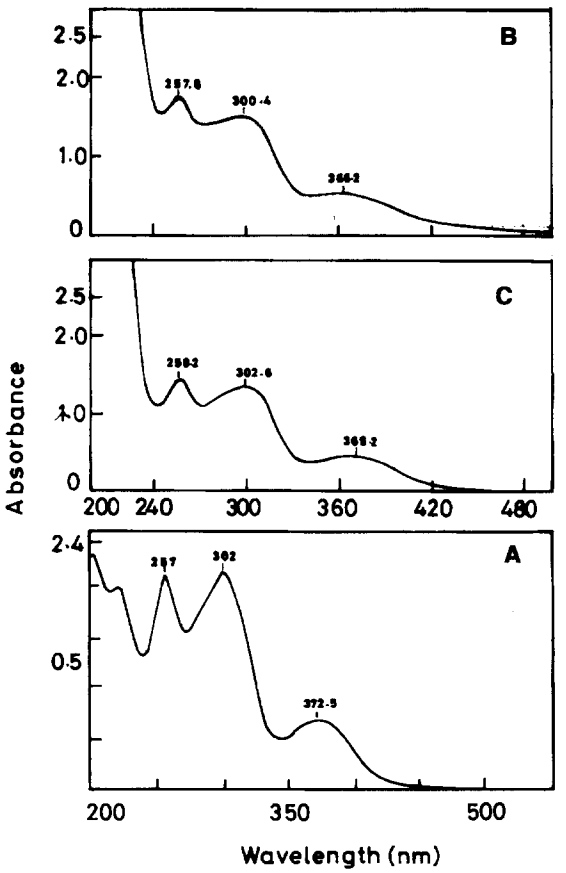

Fig. 2. Ultra violet spectra of (a) MTX; (b) GMC-I; (c) GMC-II.

respectively. The UV spectrum of MTX showed the three absorption maxima, at 257 nm, 302 nm and 372 nm. In the UV spectrum of GMC-I the peaks at 302 nm and 372 nm have shifted to 300 nm and 366 respectively. Whereas in the case of GMC-II the peaks at 257 nm and 372 nm had shifted to 258.2 nm and 368.2 nm, respectively. Gelatin by itself has no peak at these absorption maxima. These results clearly indicated the formation of conjugates of MTX and gelatin by the azide coupling method.

3.2.2. Fourier transform infra-red spectroscopy

The IR spectra was also used to establish that the conjugates were separate chemical identities different from gelatin and MTX. The FT-IR spectra of gelatin and MTX are shown in Fig. 3a, Fig.

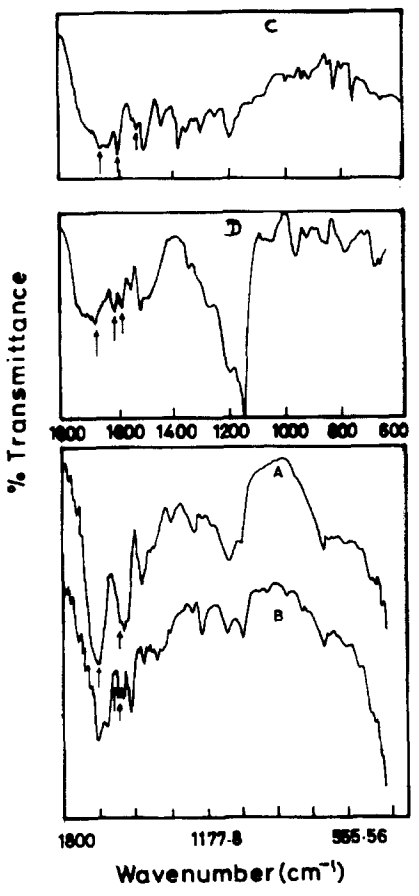

Fig. 3. Infra red spectra of (a) MTX; (b) gelatin (c) GMC-I; (d) GMC-II.

3b, Fig. 3c and Fig. 3d. The infra-red spectrum of gelatin showed the characteristic peaks of amide I and II at 1651 cm^{-1} and 1557 cm^{-1} respectively. In the infra-red spectrum of MTX, the aromatic ring stretching at 1575 cm^{-1} and secondary amide peak at 1558 cm^{-1} were seen. The IR spectra of GMC – I and GMC-II showed the amide I peaks at 1665 cm^{-1}, 1656 cm^{-1} and amide II peaks at 1550 cm^{-1} and 1541 cm^{-1} which are characteristic of gelatin. The peaks due to aromatics ring stretching of MTX were seen at 1598 cm^{-1} and 1578 cm^{-1} respectively in the case of GMC-I and GMC-II. Furthermore, when the synthetic reaction was conducted without the azide coupling method the reaction did not yield a stable conjugate but only a physical mixture of gelatin and MTX from which MTX could be rapidly dialysed out using phosphate buffer. This fact, along with the spectroscopic evidence established the formation of a covalent linkage between gelatin and MTX when the reaction was conducted following the azide coupling method. There are two carboxyl groups on the glutamic acid of MTX. In our experiments no efforts were made to know whether the azide is coupled to the α or τ COOH groups of MTX.

3.2.3. MTX content of gelatin MTX conjugates: GMC-I and GMC-II

The second peak in the gel separation chromatogram of each of the crude GMC was due to free MTX. From the initial amount of MTX and the amount of free MTX, the amount of MTX coupled to gelatin was calculated.

In the case of conjugate prepared by coupling gelatin to MTX azide, (GMC-I) 410 mg of MTX had reacted with gelatin to form the conjugate with a drug content of 205 μg/mg gelatin. Whereas in the case of GMC-II prepared by coupling MTX to gelatin azide 406 mg of MTX had reacted with gelatin to form a conjugate with drug content of 203 μg/mg gelatin (Table 1).

3.3. Gelatin microspheres containing conjugated MTX: GMCM-I and GMCM-II

GMCM-I and GMCM-II were prepared by the

Table 1
MTX content of gelatin-MTX conjugates

Type of GMC	Amount of gelatin (g)	Amount of MTX (mg)	Amount of MTX in GMC	μg MTX/mg gelatin
GMC-I	2	500	410	205
GMC-II	2	500	406	203

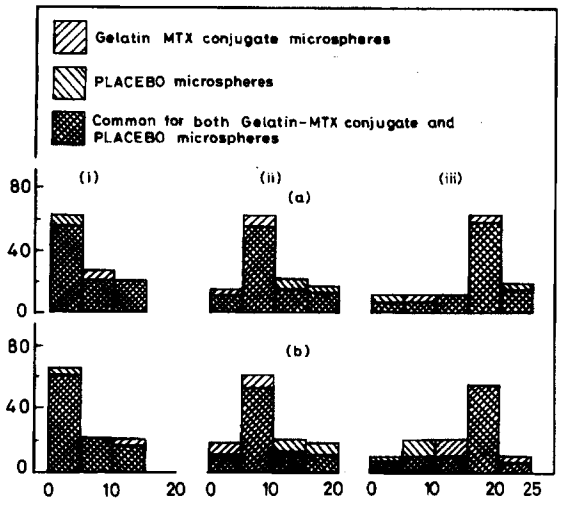

Fig. 4. Particle size distribution of (a) GMC-I and (b) GMC-II of mean particle size (i) 1–5 μm; (ii) 5–10 μm; and (iii) 10–20 μm.

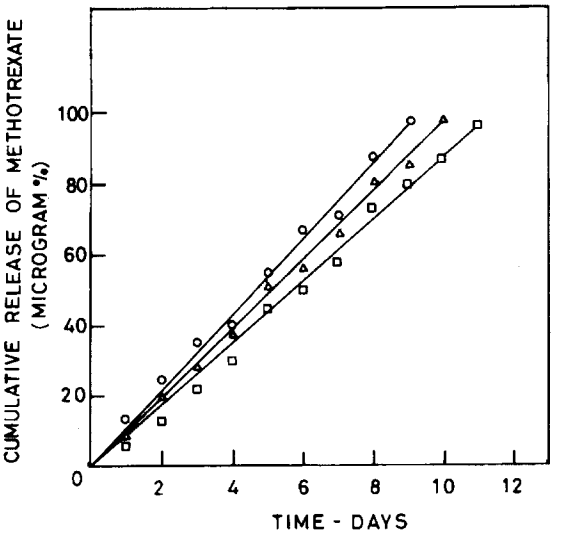

Fig. 5. In vitro release of MTX in 0.01 M PB, pH 7.4 at 37°C from GMCM-I of: (○) mean particle size 1–5 μM; (Δ) mean particle size 5–10 μM; and (□) mean particle size 15–20 μM.

novel polymer dispersion method using the conjugates GMC-I and GMC-II, respectively. The microspheres were obtained as a uniform and free flowing powder. By making appropriate changes in the concentration of PMMA, it was possible to obtain GMCM-I and GMCM-II of various mean particle size ranges.

3.4. Characterisation of GMCM-I and GMCM-II

The gelatin microspheres containing conjugated MTX (GMCM-I) were characterised for their particle size and in vitro drug rlease profiles.

3.4.1. Particle size of GMCM

The particle size of microspheres prepared using the two conjugates were in the range of 1–20 μm. However, it was possible to get microspheres of lower size ranges, i.e. 1–5 μm and 5–10 μm by selecting appropriate experimental conditions such as concentration of PMMA during the preparation of microspheres. As shown in Fig. 4, the mean particle sizes of GMCM-I and GMCM-II prepared using the two conjugates were in the range of 1–5 μm, 5–10 μm and 15–20 μm.

3.5. In vitro release studies

The in vitro release profiles of the drug were studied in simulated gastric fluid and simulated intestinal fluid.

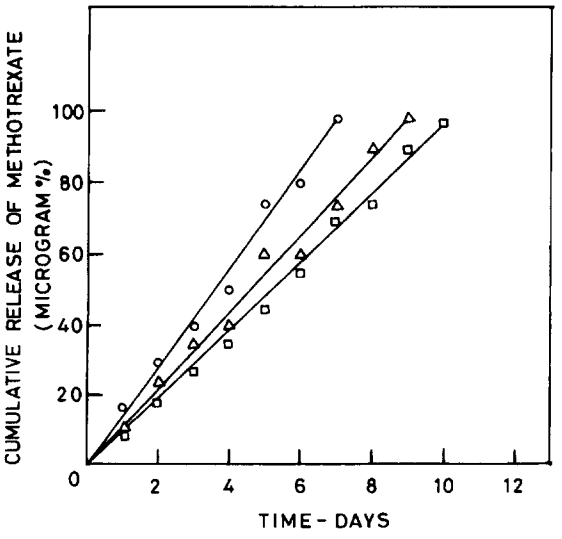

Fig. 6. In vitro release of MTX in 0.01 M PB, pH 7.4 at 37°C from GMCM-II of: (○) mean particle size 1–5 μM; (Δ) mean particle size 5–10 μM; and (□) mean particle size 15–20 μM.

Fig. 5 and Fig. 6 show the release of MTX from GMCM-I and GMCM-II in simulated intestinal fluid. It was seen that about 92–97% of MTX was released in a zero order controlled manner for 9–11 days from GMCM-I. On the other hand GMCM-II released about 97–98% of MTX for 7–10 days in simulated intestinal fluid in a controlled zero order manner. The release data also showed that the rate of release of MTX from GMCM was faster in gastric medium when compared to intestinal medium. From Figs. 5 and 6 it is clearly seen that the rate of MTX release decreases with increase in the particle size of GMCM.

3.5.1. Effect of particle size of GMCM on MTX release

The release studies also established that the rate of MTX release from large size microspheres was slower than compared to small size microspheres. This is because the rate of dissolution of the drug from small size particles is higher due to their large surface area. Therefore, release of the drug from smaller microspheres is faster when compared to larger microspheres. 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.

These results indicated that the release rate of MTX increased with decrease in the particle size of microspheres. Hence, it is possible to regulate the release of MTX for 7–10 days by using gelatin microspheres containing conjugated MTX of various size ranges.

4. Conclusion

By using the azide coupling technique, two azides namely, gelatin azide and MTX azide were prepared and subsequently coupled to MTX and gelatin respectively. The in vitro evaluation of the microspheres of different sizes prepared using the macromolecular conjugates released MTX in a controlled zero order manner for prolonged periods in simulated intestinal fluid. These studies indicated that the microspheres containing conjugated MTX developed in the present study have good potential as delivery systems for MTX and will help avoiding the toxicity associated in cancer treatment using the free drug. A cocktail of two types of microspheres containing conjugated MTX and free MTX can be used to regulate the release over a prolonged period of time. Moreover the GMCM-I and GMCM-II developed in this study can also be utilised for combination therapy of two synergistic drugs of schedule dependency such as 5-fluorouracil with MTX.

References

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

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

Hiroshi, M., Len, S.W. and Yoichi, M., Conjugates of anticancer agents and polymers: advantages of macromolecular therapeutics in vivo. *Bioconj. Chem.* 3, (1992), 351–362.

Jeong, S.Y. and Kim, S.W., Biodegradable polymeric drug delivery systems. *Arch. Pharm.*, 9(2), (1986), 63–73.

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

Kotanagi, H., Fukuda, and Ogata, N., Potent effect of the monoclonal antibody-mitomycin C conjugate on human colon cancers. *Jpn. J. Sug.*, 17, (1987), 47–50.

Li, X., Bennett, D.B., Adams, N.W. and Kim, S.W., A biodegradable injectable drug delivery system. In R.L. Dunn and R.M. Ottenbrite (Eds.), *Polymeric Drug and Drug Delivery Systems*. American Chemical Society, Washington, DC, 1990, pp. 101–116.

Mukhopadhyay, A. and Basu, S.K., Enhanced intracellular delivery of methotrexate by a receptor-mediated process. *Biotech. Appl. Biochem.*, 12, (1990), 529–636.

Narayani, R. and Panduranga Rao, K., Preparation, characterisation and in vitro stability of hydrophilic gelatin microspheres using a gelatin-methotrexate conjugate. *Int. J. Pharm.*, 95, (1993), 85–91.

Noguchi, A., Takahashi, T., Yamaguichi, T., Kitamura, K., Takakura, Y., Hasida, M. and Sezaki, H. (1992) Preparation and properties of the immunoconjugate composed of anti-human colon cancer monocolonal antibody and mitomycin C-dextran conjugate. *Bioconj. Chem.*, 3. (1992), 132–137.

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.

Shih, 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.

Takakura, Y., Kitajima, M., Matsumoto, S., Hashida, M. and Sezaki, H., Development of a novel polymeric prodrug of mitomicin C, mitomycin C-dextran conjugate with anionic charge. I. Physicochemical characteristics and in vivo antitumor activities. *Int. J. Pharm.*, 37, (1987), 135–143.