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Development of hydrophilic human serum albumin microspheres using a drug–albumin conjugate

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Summary

Methotrexate (MTX) was covalently linked to human serum albumin by the carbodiimide reaction and the conjugate was separated by the gel filtration method. Hydrophilic drug-conjugated albumin microspheres were prepared by using the conjugate and then modifying the surface of microspheres with carboxyl groups. Hydrophilic albumin microspheres containing conjugated drug (HAMC) were easily dispersed in aqueous medium without the need of surfactants, and the dispersed system remained stable enough for the injectable suspension. As compared with hydrophilic albumin microspheres entrapping free drug (HAMF), drug incorporation efficiency greatly increased and matrix degradation by protease delayed. The decrease of burst out effect was also observed. In case of HAMF, drug release by protease was proportionally increased from the initial incubation time, whereas MTX release from HAMC began to increase progressively after several hours. The characteristic drug release pattern of HAMC suggests that the amount of drug released might be effectively controlled over more prolonged period by the combined usage of HAMC and HAMF mixed in previously designed ratio. Further, it is expected that this concurrent administration of HAMF and HAMC may be applied to the combination chemotherapy of two synergistic drugs with schedule dependency.

Introduction

Delivery of chemotherapeutic agents to target sites at proper rate and amount constitutes one of the major challenges of chemotherapy. For this purpose, many attempts have centered around the development of drug delivery systems such as albumin microspheres (Kim et al., 1985), liposomes (Kim et al., 1987), and conjugates between drugs and polymers including polysaccharides, natural proteins and polypeptides (Zunino et al., 1984).

Of these systems, albumin microspheres have received wide attention because of their specific organ targeting, biocompatibility and other required characteristics of ideal drug carriers (Yapel, 1985; Kim et al., 1985). It has been reported that drug release from albumin microspheres may be controlled by their cross-linking degree and drug/albumin ratio during preparation (Kim et al., 1986). However, the control range of drug release through only these methods has been limited by some drawbacks. At first, the microspheres pre-

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pared at highly hardened conditions are often too slowly degraded to release a therapeutically effective amount of drug. The second control method by drug/albumin ratio has its limiting factors in physicopharmaceutical properties of drug, such as solubility in albumin solution. Further, although drug was entrapped in microspheres to a great extent, large percentages of incorporated drug are usually lost by a burst-out effect in the initial period.

On the other hand, Longo et al. (1985) reported the advantage of albumin microspheres which had hydrophilicity required for easy wetting and stable suspension.

It is thus necessary to develop a novel type of hydrophilic albumin microspheres capable of mitigating such a large burst-out effect while extending control capacity of drug release over more prolonged periods. Therefore, in this study, hydrophilic drug-conjugated albumin microspheres were developed with methotrexate-albumin (MTX-HSA) conjugate, and their physicopharmaceutical properties were examined.

Materials and Methods

Materials

MTX sodium was kindly supplied by Yu Han Pharm. Co. (Seoul, Korea). Human serum albumin (HSA) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) were purchased from Sigma Chemical Co. (St. Louis, MO). Sephadex G-50 was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). All other chemicals were of commercial reagent grade.

Synthesis of MTX-HSA conjugates

100 mg of HSA was dissolved in 20 ml of distilled water. After the pH of HSA solution was adjusted to 4.7 with 0.1 N HCl, 25 mg of MTX and 150 mg of EDC were added. The reaction was allowed to proceed for 6 h at room temperature. The resultant solution was loaded on Sephadex G-50 column (2.5 × 30 cm). Then, macromolecular fractions were pooled and lyophilized.

Determination of MTX / HSA ratio in the conjugate

The ratio of MTX to HSA in the conjugate was calculated from the absorbance at 370 nm for determining MTX and at 540 nm for measuring HSA concentration by the Biuret method (Layne, 1957).

Preparation of albumin microspheres

Hydrophilic albumin microspheres containing conjugated drug (HAMC) were prepared by combining the basic preparation method (Scheffel et al., 1972) and the surface modification concept (Longo et al., 1985) as follows. 1 ml of MTX-HSA conjugate solution (10% w/v) containing 4 mg of MTX as conjugated form was dispersed in 20 ml of cottonseed oil. The resultant emulsion was diluted to 50 ml with cottonseed oil and homogenated at 1700 rpm for 20 min. This solution was placed in an ice bath and further emulsified by an ultrasonicator (Branson Cleaning Equipment Co., CN). Then, as a cross-linker, glutaraldehyde was added to the fine emulsion containing MTX-HSA conjugate which had been stirred at 1700 rpm. After cross-linking the reaction proceeded for the required time, 1.5 ml of glycine solution (200 mg/ml) was added and further stirred for 1 h. The products were washed with anhydrous diethyl ether and then distilled water. Fine HAMC powder was obtained by freeze-drying. Hydrophilic albumin microspheres containing free MTX (HAMF) were prepared by the same method, but using HSA solution (10% w/v) containing 4 mg of free MTX. Cross-linked albumin microspheres (CAM) without surface modification were made under the same hardening condition with HAMC, but not treated with glycine.

Studies of shape and size distribution

The lyophilized microspheres were vacuum-coated with gold with an ion coater. The shape and size distribution of microspheres were examined by scanning electron microscopy (JEOL JSM-35, Jeol Ltd.).

Measurement of hydrophilicity

A quantitative capillary rise technique (Longo et al., 1985) was used to evaluate the hydrophilicity of the newly developed microspheres.

Three capillaries of the same inner diameter (1.2 mm) were packed with HAMC, HAMF, and the surface non-treated CAM. These capillaries were placed in a flat-bottomed dish containing distilled water as an aqueous mobile phase. The rates of water rising were examined over 60 min.

Suspension stability test

The stability of microsphere dispersed suspension system was assessed by the sedimentation volume method (Lachman et al., 1986). A microsphere dispersion in 10 ml mass cylinders remained undisturbed and the changes of sedimentation volume were estimated at proper time intervals.

Assessment of drug incorporation

The amount of MTX entrapped in microspheres was estimated by digesting microspheres with protease. 2 mg of microspheres dispersed in 60 ml of phosphate buffer (pH 7.4) were incubated at $37 \pm 2°$ C and degraded under the reaction of protease (63 units/ml). Then, the solution was deproteinized with 5% trichloroacetic acid (TCA), and amount of MTX was determined by the absorbance of the filtrate at 370 nm by UV-spectrophotometer (Ultrospec 4050, LKB).

In vitro drug release

After 4 mg of microspheres were dispersed in 80 ml of phosphate buffer (pH 7.4), the suspension was maintained at $37 \pm 2°$ C, and constantly stirred at 300 rpm. The samples taken at scheduled intervals were filtered and spectrophotometrically analyzed at 370 nm.

Effect of protease on in vitro drug release

To fine suspension of microspheres in $37 \pm 2°$ C water bath, protease was added. At various time intervals, 4 ml of the sample solution was collected and deproteinized with 5% TCA. The amount of released MTX was calculated by the absorbance of filtrate at 370 nm.

Matrix degradation study

The measurement of matrix degradation was based on the fact that the turbidity of particle suspension varies with the particle size change (Campbell et al., 1984). The homogeneous suspension containing 5 mg of dispersed microspheres was incubated at $37 \pm 2°$ C in the presence of 80 mg protease. The samples were collected at proper time interval and their turbidity was measured at 600 nm.

Results and Discussion

Conjugation of MTX-HSA

MTX was conjugated to HSA by using EDC as a hydrochloride form. The carbodiimide compound, EDC was selected as a coupling agent, since it is water-soluble and reactive in aqueous mild conditions. In order to characterize the linkage bond of the conjugate, the drug elution pattern of the carbodiimide-reacted system was compared with that of physical mixture of MTX and HSA. As shown in Fig. 1, when the physical mixture of MTX and HSA was loaded, almost the entire amount of MTX was detected at the small molecular fractions, whereas in the case of the EDC-catalyzed system, the high optical density of MTX was observed at the macromolecular fractions as well as at the small molecular fractions. In view of these results, it seems that MTX detected at the macromolecular fraction pool existed as the conjugated form covalently bound to HSA by the carbodiimide.

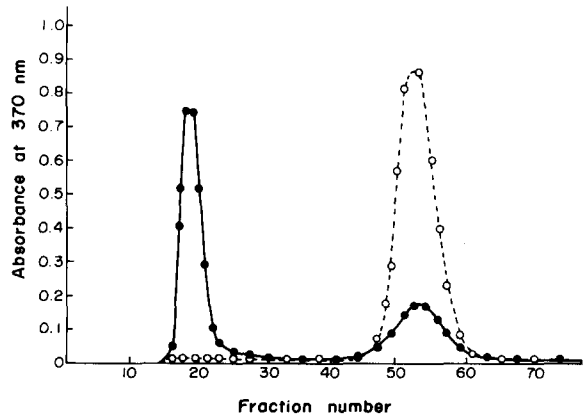

Fig. 1. Gel filtration patterns on a Sephadex G-50 column eluted with 0.05 M phosphate buffer. ●———●, HSA + MTX + EDC; ○------○, HSA + MTX.

TABLE 1

The size of drug-conjugated albumin microspheres vs cross-linking time during preparation

Cross-linking time (min)	Mean diameter (μm)	S.D.
30	1.61	0.790
60	1.59	0.488
90	1.46	0.455

Shape and size distribution of microspheres

HAMC using MTX-HSA conjugate were spherical and showed nearly the same mean diameters regardless of cross-linking time. But the standard deviation of microsphere size tended to diminish with increase of cross-linking time (Table 1).

Evaluation of hydrophilicity of microspheres

The capillary rising velocities of HAMC and HAMF were compared with that of CAM without being treated with glycine solution.

Fig. 2 demonstrates that the hydrophilicity of microspheres was remarkably enhanced by the surface modification with the carboxyl groups of glycine, which replaced the reactive aldehyde groups on the surface of microspheres.

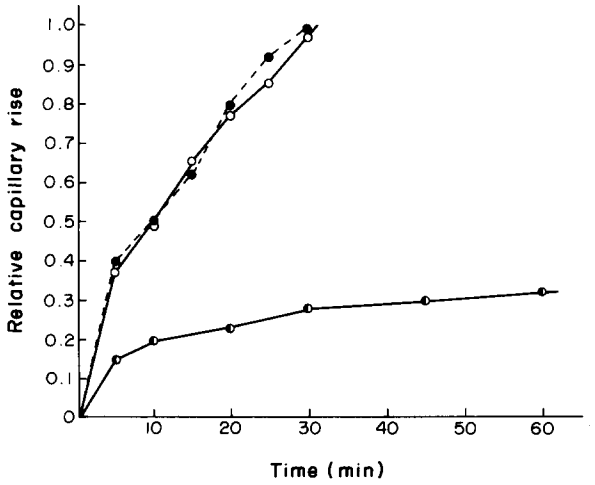

Fig. 2. Capillary rise degree of 3 types of albumin microspheres according to time. ○———○, HAMF; ●------●, HAMC; ◐———◐, CAM. All albumin microsphere samples were prepared by cross-linking for 60 min. HAMF and HAMC were further treated with glycine solution.

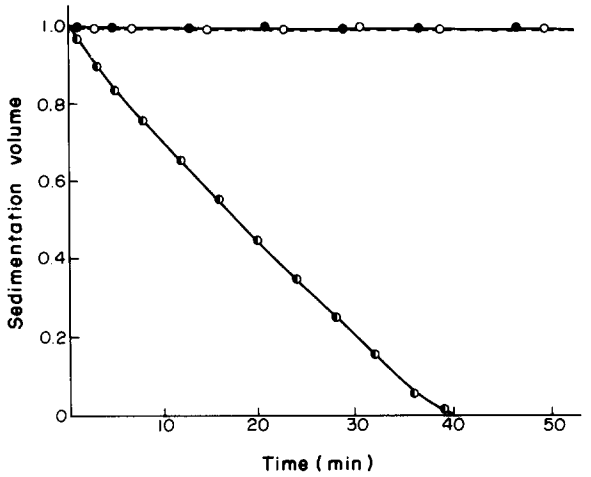

Fig. 3. The sedimentation volume of various human serum albumin microspheres vs time. ⊙------⊙, HAMF; ●———●, HAMC; ◐———◐, CAM.

Stability of microsphere-dispersed suspension

The physical stability of microsphere-dispersed suspension system was evaluated by measuring the sedimentation volumes which considered the ratio of the height of the sediment at any particular time (H_t) to the initial height (H_0) of the total suspension as a function of settling time. The more horizontal the line, the better is the stability of the suspension.

In Fig. 3, the time plot of sedimentation volume of HAMC and of HAMF showed almost horizontal lines for more than 50 min, whereas the time plot of sedimentation volume of cross-linked albumin microspheres without surface modification (CAM) produced the steep line and sedimented completely within 40 min. These results revealed that CAM-dispersed suspension, which formed microsphere cake quickly, is not adequate for injection while suspensions of the surface-modified hydrophilic microspheres are stable enough for injectable suspension. Further, the hydrophilic albumin microspheres can solve the problem of blood embolism, one of the drawbacks of relatively hydrophobic microspheres.

TABLE 2

Relationship between the preparation condition of albumin microspheres and the amount of entrapped MTX (μg/mg microspheres)

Cross-linking time (min)	Entrapped MTX	
	HAMF	HAMC
30	11.0	26.1
60	12.5	30.5
90	10.2	31.4

Drug incorporation efficiency

The amounts of MTX entrapped in HAMC and HAMF are summarized in Table 2. The amount of drug entrapped in HAMC increased more than two times as compared with that in HAMF. This seems to be caused by the MTX that was covalently bound to the albumin matrix, and is lost to a lower extent than free MTX during the preparation of microspheres. This improved incorporation efficiency of HAMC has an advantage, in the sense that it can make high concentrations of drug carried in fewer microspheres while minimizing the problematic side effect of high-dose chemotherapy.

In vitro drug release

Three types of HAMC prepared by varying cross-linking time, 30 min, 60 min and 90 min were tested to examine the influence of chemical cross-linking degree on the in vitro drug release.

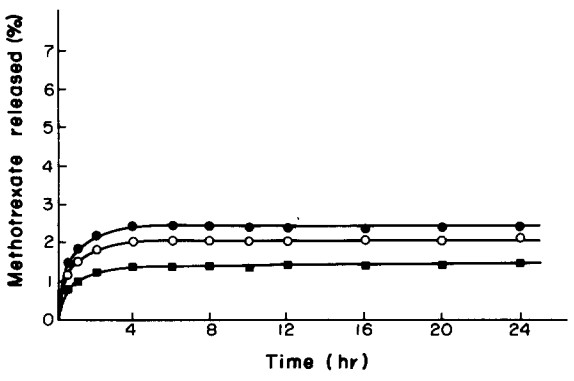

Fig. 4. In vitro release of methotrexate from HAMC. ●———●, 30 min; ○———○, 60 min; ■———■, 90 min.

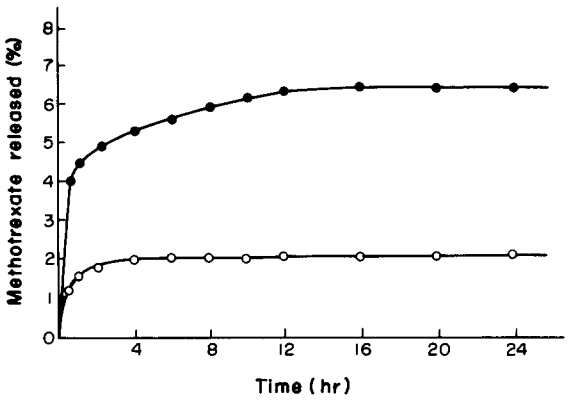

Fig. 5. In vitro release of drug from HAMC compared to that of HAMC. ●———●, HAMF; ○———○, HAMC. All microsphere samples were cross-linked for 60 min.

From Fig. 4, it could be expected that MTX was not released any more after 4 h had elapsed, and the released amounts were slightly changed by the cross-linking degree. Fig. 5 also shows that MTX entrapped in free form was continuously released from the microspheres as much as 6% of the total entrapped amount by 12 h, while the released drug from HAMC was at most 2%.

It is thought that drug release from HAMC depends predominantly on the slow hydrolysis of drug covalently attached in the near surface of microsphere, whereas drug release from HAMF proceeds with the fast release of physically associated drug in albumin matrix (Kim et al., 1986).

Matrix degradation and drug release by protease

The matrix of HAMC was slowly degraded by protease and the degradation rate was retarded as the hardening time during preparation was increased (Fig. 6). $T_{1/4}$, that is the time passed for degrading 25% of matrix by protease, was measured for comparing the hardness of each microsphere prepared at different conditions. The $T_{1/4}$ values of HAMC were increased by almost similar degree, average 8.5 times, when compared with those of HAMF prepared at the same condition (Table 3). These findings are probably due to the increased compactness of HAMC matrix, and the enhanced hindrance in enzymatic digestion on the drug-conjugated albumin matrix of HAMC in

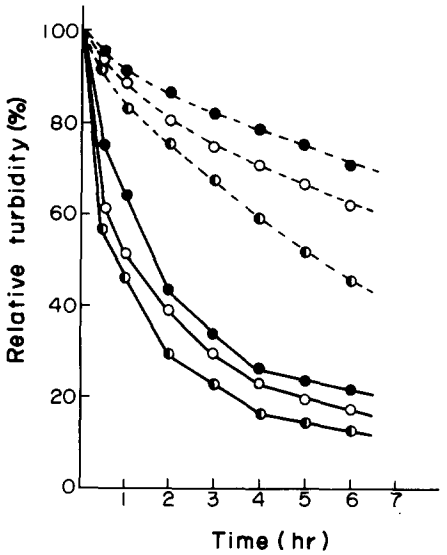

Fig. 6. Matrix degradation of albumin microspheres prepared at different hardening conditions. ◐———◐, HAMF 30 min; ◐------◐, HAMC 30 min; ○———○, HAMF 60 min; ○------○, HAMC 60 min; ●———●, HAMF 90 min; ●------●, HAMC 90 min.

comparison with that on the free albumin cross-linked matrix of HAMF.

Fig. 7 shows that the longer duration of cross-linking increased the lag time, and delayed the release of MTX from HAMC. It is thus likely that the change of lag time can be accompanied by the change of cross-linking degree affecting microsphere porosity, hydration and swelling (Longo et al., 1985). However, the drug release pattern from HAMC and HAMF which were prepared under the same cross-linking condition showed distinctive contrast (Fig. 8). MTX release from HAMF became increased in proportion to time from the

TABLE 3

Matrix degradation as a function of hardness of various type albumin microspheres

Hardening time (min)	$T_{1/4}$		
	HAMF (min)	HAMC (min)	Ratio HAMC/HAMF
30	14	118	8.4
60	20	172	8.6
90	33	286	8.7

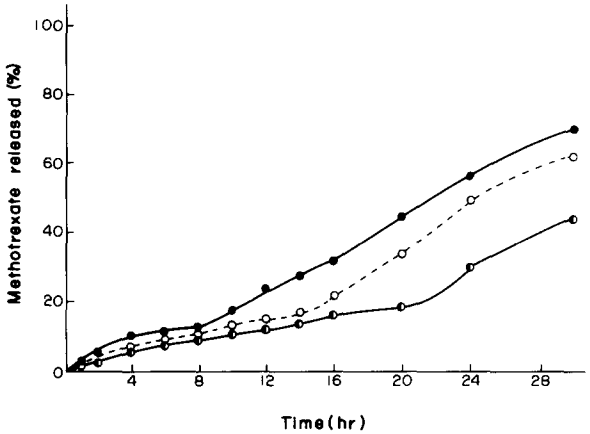

Fig. 7. Effect of hardening time on the drug release from HAMC incubated with protease. ●———●, 30 min; ○------○, 60 min; ◐———◐, 90 min.

first, whereas MTX in HAMC began to be gradually released after several hours had elapsed. It is also shown that the cross-linking time of HAMC only affected the lag time, and after each lag time, MTX release from 3 types of HAMC with different cross-linking densities were processed with nearly the same rates (Fig. 7). These results indicate that drug release from HAMC may be determined not only by cross-link density, but also by another factor which seems to be the covalent linkage of conjugated drug. From our experi-

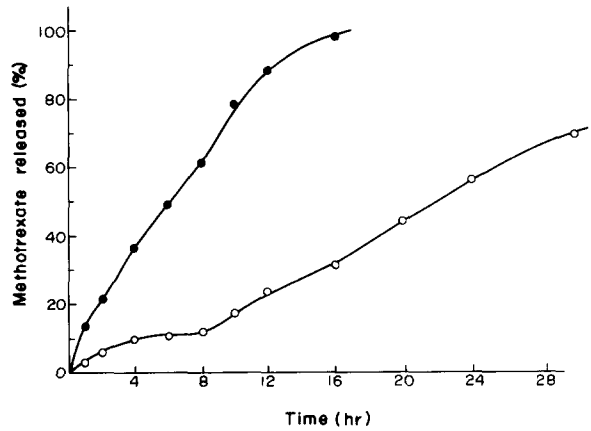

Fig. 8. The difference of methotrexate release pattern between HAMF and HAMC tested by incubation in the presence of proteolytic enzyme at 37°C. ●———●, HAMF; ○———○, HAMC.

ments, it seems probable that the first rate-determining step is hydration and swelling of microspheres which enable the reaction of protease on microsphere matrix, and the second rate-determining step is degradation of the covalent bond between drug and albumin matrix.

On the basis of these significant results, it is suggested that the control of MTX release from microspheres over prolonged periods could be achieved by using microspheres entrapping conjugated MTX together with microspheres containing free MTX. Furthermore, it is expected that this concomitant administration of HAMC and HAMF may be applicated to the combination therapy of two synergistic drugs with schedule dependency, for example MTX and 5-fluorouracil, by the simultaneous administration of HAMF entrapping free drug such as MTX, and HAMC containing conjugated other drugs. Therefore, the present investigation has shown the potential of HAMC as a useful drug delivery system especially in liver cancer chemotherapy. In vivo studies have been carried out in this laboratory and will be published in the near future.

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