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Preparation and *In vitro* Release Characteristics of Hydrophilic Albumin Microspheres Containing Methotrexate and Methotrexate-Human Serum Albumin Conjugates

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Abstract □ Release characteristics of five different types of hydrophilic albumin microspheres (HAM) containing different ratios of methotrexate-albumin (MTX-HSA) conjugates to free MTX; 1 : 0 (HAMC), 3 : 1 (HAMC3F), 1 : 1 (HAMCF), 1 : 3 (HAMCF3) and 0 : 1 (HAMF) were investigated in the absence or presence of protease using dissolution tester. In all the HAMs studied except HAMC, the MTX was released bi-exponentially in the absence of protease; an initial fast release period up to approximately 6 h, and thereafter the release rate was very much slower. The fast release of MTX from the HAMs (such as HAMC3F, HAMCF, HAMCF3 and HAMF) at the initial phase is probably due to the release of "physically associated" MTX on the surface and/or entrapped in the near inner surface of HAMs and the slow release at the second phase is due to the release of entrapped free MTX from the core of the HAMs. The initial rate constants were 7.2, 8.7, 8.5 and 5.9 times greater than the second rate constants for HAMF, HAMCF3, HAMCF and HAMC3F, respectively. MTX release from HAMC was very slow and mono-phasic. It was at most 2.2% of the total entrapped amount by 24 h. The protease accelerated the release of MTX from the HAMs. The percentages of MTX released from HAMs up to 24 h were 100, 89.0, 75.0, 66.0 and 61.0% for HAMF, HAMCF3, HAMCF, HAMC3F and HAMC, respectively in the presence of protease and the corresponding values in the absence of protease were 30.2, 19.0, 10.0, 6.5 and 2.2%, respectively. *In vitro* release of MTX in the presence of protease varied according to the ratios of MTX-HSA conjugates to MTX; the data set from HAMF, HAMCF3 and HAMCF fits better to monophasic first-order profile more adequately than to zero-order profile, that of HAMC3 F to mono-phasic zero-order, and that of HAMC to bi-phasic zero-order. Above results suggested that zero-order release rate can be achieved by adjusting the ratio of MTX-HSA conjugates to MTX in the preparation of HAMs such as HAMC3F.

Keywords □ Hydrophilic albumin microspheres; methotrexate; methotrexate-human serum albumin conjugates, *in vitro* drug release, drug release by protease, zero-order release.

Albumin microspheres have been proposed as a drug delivery system for the targeting of anticancer drugs to various organs and tissues[1], and for sustained release of drug at the targeted sites[2,3]. In most of previous studies, microspheres have incorporated only free drug and thus have some following drawbacks; low drug incorporation efficiency, narrow control range of drug release and a burst-out effect in the initial period of release[4-6]. Hence, it was required for improving the above drawbacks in the microspheres. Theoretically, the increase of drug incorporation efficiency in the microspheres can not only ensure delivery of drug in high concentrations at target cells, but also alleviate toxic effects of the microspheres themselves, if any, by reducing the total amounts of carrier adminisered per dose[7,8]. Anticancer drug-albumin conjugates have been also proposed as a good drug delievery system for cancer chemotherapy[9-13]. It provoked us to prepare a no-

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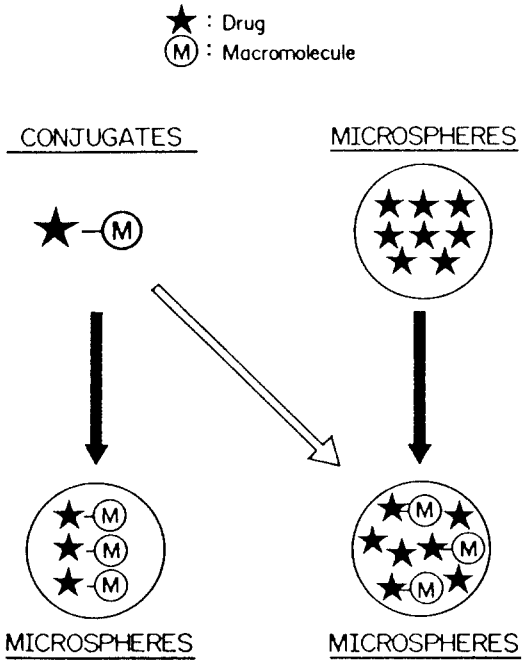

Fig. 1. Model for albumin microspheres encapsulated with free drug and/or drug-macromolecule conjugates.

vel type of hydrophilic albumin microspheres (HAM) with drug-albumin conjugate capable of mitigating such a large burst-out effect while extending control capacity of drug release over prolonged periods of time. Therefore, HAMs entrapping different ratios of MTX-HSA conjugates to MTX might be a good drug delivery system for the chemotherapeutic agent than that of HAMs entrapping only free MTX as shown in Fig. 1.

In this paper, five diferent types of HAM containing different ratios of MTX-HSA conjugates to MTX were prepared and their *in vitro* release characteristics were studied.

EXPERIMENTAL METHODS

Materials

MTX was kindly supplied by the Choong-Wae Pharm. Co. (Seoul, Korea), and Human serum albumin (Fraction V) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC, Protein sequencing reagents) were products of the Sigma Chemical Co. (St. Louis, MO, USA). Sephadex® G-75-40 (particle size: 10-40 μm) was obtained from the Pharmacia Fine Chemicals (Uppsala, Sweden) and Spectrapor® membrane tubing (m.w. cut off: 6,000-8,000, cylinder diameter: 14.6 mm) was purchased from the Spectrum Medical Ind. (Terminal Annex, Los Angeles, CA, USA). All other chemicals were of reagent grade and used without further purification.

Synthesis of MTX-HSA and determination of MTX/HSA ratios in the conjugates

MTX-HSA conjugates were prepared by the slight modification of the reported method[7,9,10]. In brief, after MTX (30 mg, dissolved in 3 ml of 0.01 N NaOH) was added to HSA (150 mg, dissolved in 10 ml of distilled water), EDC (150 mg, dissolved in 3 ml of 0.05 N HCl) was added slowly to the above solution at pH 5.0-6.0 for 7 h. The reaction mixture was kept overnight at 4℃. The resultant solution was loaded on Sephadex G-75-40 column (2.5×30 cm). The first conjugate fraction (dimers or polymers) was discarded and the remaining macromolecular fractions were pooled. Before lyophilization, macromolecular fraction was dialyzed by Spectrapor® membrane tubing to remove buffer components and the other small molecules. The contents of MTX in MTX-HSA conjugates were found to be 90-111 mg per g of the conjugates when measured by the method described previously[7].

Preparation of HAMs containing different ratios of MTX-HSA conjugates to MTX

HAMs containing different ratios of MTX-HSA conjugates to MTX were prepared by the chemical cross-linking and the surface modification technique reported previously[7]. Five types of HAM were prepared from the different ratios of MTX-HSA conjugates to free MTX; 1:0 (HAMC), 3:1 (HAMC3F), 1:1 (HAMCF), 1:3 (HAMCF3) and 0:1 (HAMF). Total amounts of MTX and/or MTX-HSA conjugates used in the preparation of HAMs were equivalent to 4 mg of MTX per 100 mg of HSA. The hydrophilicity of the HAMs prepared by the surface modification technique was evaluated from our laboratory[7] using capillary rise technique[14].

The amounts of MTX entrapped in the HAMs containing MTX and/or MTX-HSA conjugates were estimated by the reported method[7] after digesting

the HAMs with protease.

Particle size analysis of HAMs containing different ratios of MTX-HSA conjugates to MTX

Particle size of HAMs, which are suspended in 0.9% NaCl solution (ISOTON® II, Coulter Electronics Ltd., Luton, Beds, U.K.), was measured by the Coulter Multisizer® (Coulter Electronics Ltd.) with 70 μ or 140 μ aperture. The mean diameter was determined from the particle size distribution of the HAM on a number- or a volume-basis. The size distribution (polydispersity) was measured in terms of a SPAN factor expressed as:

$$SPAN = \frac{D_{90\%} - D_{50\%}}{D_{50\%}}$$

where $D_{90\%}$, $D_{50\%}$ and $D_{10\%}$ are the diameters where the given percentage of particles is smaller than that size[15]. A high value of SPAN indicates a wide distribution in size and a high polydispersity.

In vitro release of MTX from five types of HAMs

A dissolution tester (DST-200, Fine Scientific Inst., Seoul, Korea) consisting of drive assembly with two spindles was used for *in vitro* release test. Cylindrical glass vessel (120 m*l*) and two blade glass paddle were used. After 10 mg (20 mg was used in the presence of protease) of each HAM was dispersed in 80 m*l* of pH 7.4 isotonic phosphate buffer in the absence or presence of 80 mg of protease (31,400 units/g, kindly supplied by Dong-A Pharm. Co., Seoul, Korea), the glass vessel was maintained at 37± 1℃ under constant stirring (100± 1 rpm). The samples taken at scheduled intervals were filtered with 0.42 μm membrane filter (Millipore Corp., Bedford, MA, USA) Ten % trichloroacetic acid, 1.5 m*l* was added to 3 m*l* of sample containing protease in order to deproteinize, and supernatant was used for the assay of MTX. The concentration of MTX were measured using spectrophotometer at 370 nm.

Analysis of data from release test

For all sets of data from release test, percentages of MTX remained in HAMs ($P=(Q_o-Q)/Q_o\times 100$) were plotted as a function of time according to the two relationships being considered, i.e. log P versus time, and P versus time, as reported[16], where Q is the amount of drug released at time t and Q_o is the initial amount of drug present in the matrix. The data were fitted using linear regression to generate release rate constants.

Table I. The contents of MTX entrapped in hydrophilic albumin microspheres and incorporation efficiencies

HAMs	Entrapped MTX (μg per mg of microspheres)	Incorporation efficiency, %
HAMC	39.2	98.0
HAMC3F	34.8	87.0
HAMCF	30.6	76.5
HAMCF3	26.8	67.0
HAMF	21.8	54.5

RESULTS AND DISCUSSION

Drug incorporation efficiency of HAMs containing MTX and/or MTX-HSA conjugates

The amounts of MTX entrapped in the HAMs are summarized in Table I. In general, the amount of MTX entrapped in the HAMs increased with the increasing ratios of MTX-HSA conjugates to MTX; the entrapment of MTX increased 79.8, 59.6, 40.4 and 22.9% for HAMC, HAMC3F, HAMCF and HAMCF3, respectively when compared with the value of HAMF. The incorporation efficiencies (% of MTX entrapped into the HAMs when compared to the total amounts of MTX added in the preparation of the HAMs) were 98.0, 87.0, 76.5, 67.0 and 54.5% for HAMC, HAMC3F, HAMCF, HAMCF3 and HAMF, respectively. Total amount of drug incorporated in HAMs was usually dependent upon physical properties of drug (i.e., solubility, partitioning of drug into the oil or washing media), cross-linking density and preparation parameters (i.e., temperature, stirring rate, shape of blade, etc.). The results in Table I seem to be affected by physical properties of MTX such as molecular weight, solubility, partitioning of MTX into the oil or washing media during the preparation of HAMF and albumin binding capacity. MTX is known to bind physically to the extent of about 46% at normal clinical drug levels[17]. Thus, it was possible for large amount of unbound MTX to be diffused out to the washing medium during the preparation under current experimental conditions. Only 54.5% of free

MTX was physically entrapped in HAMF, but the drug incorporation efficiency of HAMs increased with increasing the ratio of conjugate to free MTX (HAMF vs HAMCF, HAM3F and HAMC). The drug incorporation efficiency was 98.0%, only when conjugate was used for the preparation of HAM. It might be due to the fact that all MTX in the conjugate was covalently bound to HSA as constituents of HAMC. In general, free MTX was physically incorporated in albumin matrix and MTX-HSA conjugate was existed as part of albumin matrix. The modification of the physicochemical characteristics of MTX in MTX-HSA conjugates such as the solubility, the partitioning of the drug into the oil or washing media during the preparation of the HAMs could also contribute to the increased entrapment of MTX-HSA conjugate in HAMs[18]. The loss of MTX-HSA conjugates might be reduced due to their large molecular size and cross-linking reactivity when compared to that of free MTX during the preparation of the HAMs. This high incorporation efficiency has an advantage; it can make high concentrations of drug carried in fewer microspheres while minimizing the problematic side effect caused by high-dose administration[7].

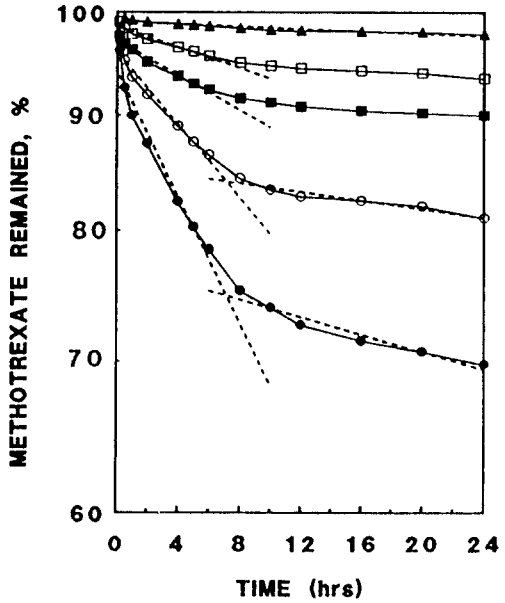

Fig. 2. *In vitro* release of methotrexate from five types of hydrophilic albumin microspheres in dissolution tester containing 0.05 M phosphate buffer (pH 7.4) kept at 37± 1°C and at the rate of 100 rpm in the absence of protease.
●—● HAMF; ○—○ HAMCF3; ■—■ HAMCF; □—□ HAMC3F; ▲—▲ HAMC.

Size distribution of HAMs

The mean diameter and polydispersity (SPAN)[15] on the number- and volume-basis of the HAMs are summarized in Table II. The volume distribution of the HAMs is more important than the number distribution, because the volume of microspheres is more closely related to the delivered amounts of microspheres to the target organ. The mean diameters on the volume- and number-basis were 20.9 ± 7.07 and 4.47± 0.618 μm, respectively for the present 5 HAMs. There was a trend that the mean diameters on volume- and number-basis tended to be decreased when MTX-HSA conjugates were not incorporated into the HAMs. However, the values of SPAN seemed to be similar regardless of the ratios of MTX-HSA conjugates to MTX.

In vitro release from HAMs containing MTX and/or MTX-HSA conjugates

Fig. 2. shows the percentages of MTX released from the five types of HAMs containing different ratios of MTX-HSA conjugates to MTX in pH 7.4 isotonic phosphate buffer solution at 37± 1℃. As expected, the release rate of MTX from the HAMs decreased with the increasing ratios of MTX-HSA conjugates to MTX, because the bond between MTX-HSA conjugates should be broken first in order to release MTX from the conjugates; the percentages of MTX released for up to 24 h from the HAMF, HAMCF3, HAMCF, HAMC3F and HAMC, were 30.2, 19.0, 10.0, 6.5 and 2.2%, respectively. In all the HAMs studied except HAMC, the HAMs displayed an initial fast release period of MTX up to approximately 6-8 h, and thereafter the release rate was very much slower. The rate constants determined from the release tests are listed in Table III. The initial rate constants are 7.2, 8.7, 8.5 and 5.9 times greater than the second rate constants for HAMF, HAMCF3, HAMCF and HAMC3F, respectively.

The release of free MTX from albumin microspheres is dependent on the location of the drug in the carrier as well as on the properties of the microsphere matrix[12,16]. Free MTX might be incor-

Table II. Particle size and SPAN (polydispersity) on the volume- and number-basis of the HAMs

HAMs	Volume basis			Number basis		
	Mean diameter(μm)	$D_{90\%}-D_{10\%}$	SPAN	Mean diameter(μm)	$D_{90\%}-D_{10\%}$	SPAN
HAMC	28.0	32.0	1.14	5.10	12.3	2.43
HAMC3F	30.6	44.4	1.45	4.39	7.63	1.74
HAMCF	18.0	20.4	1.13	5.12	10.3	2.01
HAMCF3	15.0	17.5	1.17	4.32	9.39	2.17
HAMF	13.0	14.2	1.09	3.44	8.19	2.38
Mean± SD[a]	20.9± 7.07	25.7± 11.1	1.20± 0.130	4.47± 0.618	9.56± 1.65	2.15± 0.253

[a]Standard deviation

Table III. *In vitro* release rate constant of methotrexate from HAMF, HAMCF3, HAMCF, HAMC3F and HAMC in the absence of protease

HAMs	First-order rate constant± S.E.[a](h^{-1})	
	Initial	Second
HAMF	0.0323 ± 0.00263 (0.9679)[b]	0.00451± 0.000583 (0.9461)
HAMCF3	0.0188 ± 0.00146 (0.9706)	0.00216± 0.000286 (0.9346)
HAMCF	0.00896 ± 0.000613 (0.9718)	0.00106± 0.000157 (0.9188)
HAMC3F	0.00534 ± 0.000508 (0.9567)	0.000900± 0.0000761 (0.9723)
HAMC	0.000620± 0.0000752[c] (0.8724)	–

[a]Standard error of the mean
[b]Coefficient of determination (r^2)
[c]HAMC displayed a monophasic 1st-order release

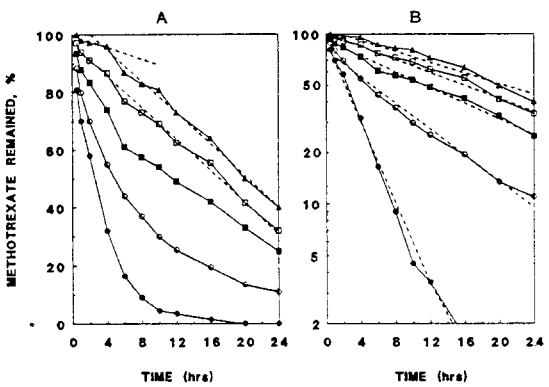

Fig. 3. Effect of protease on the release of methotrexate from 5 types of hydrophilic albumin microspheres in dissolution tester containing 0.05 M phosphate buffer (pH 7.4) kept at 37± 1°C and at the rate of 100 rpm in the presence of protease.
A is the plots of percentages of MTX remained in HAMs and B is the semilogarithmic plots of percentages of MTX remained in HAMs, respectively.
●—● HAMF; ○—○ HAMCF3; ■—■ HAMCF; □—□ HAMC3F; ▲—▲ HAMC.

porated by either adsorption onto the particle surface and/or inclusion in the microsphere matrix[16]. In Fig. 2, it is of interest to note that the release of MTX from the HAM containing only free MTX (HAMF) displayed a bi-phasic process; the fast release of MTX at the initial phase is probably due to the release of drug from the near surface of the HAMs, and the slow release at the second phase is due to the release of the entrapped drug from the core of the HAM as proposed by release characteristics of adriamycin from albumin microspheres[16]. On the other hand, MTX release from the HAMC containing only MTX-HSA conjugates was very slow and mono-phasic. It is thought that the release of MTX from MTX-HSA conjugates in the microsphere matrix depends predominantly on the slow hydrolysis of the conjugated MTX. However, the released MTX from HAMC was at most 2.2% of the total entrapped amount by 24 h. It indicated that MTX detatched from the surface of HAMC was small and almost negligible. The data presented in Fig. 2 demonstrate the release of MTX from the HAMs except HAMC is a biphasic process, the fast release of MTX at the initial phase is probably due to the release of "physically associated" MTX

Table IV. Effect of protease on the *in vitro* release of methotrexate from HAMF, HAMCF3, HAMCF, HAMC3F and HAMC in the presence of protease

Type	Adequate model employed	Rate constant ± S.E[a]
HAMF	First-order	0.272± 0.00937 h^{-1} (0.9906)[b]
HAMCF3	First-order	0.0915± 0.00397 h^{-1} (0.9815)
HAMCF	First-order	0.0537± 0.00189 h^{-1} (0.9877)
HAMC3F	First-order	0.0444± 0.00221 h^{-1} (0.9782)
	Zero-order[c]	0.944 ± 0.0278 $\mu g \cdot h^{-1}$ (0.9929)
HAMC	First-order	0.0364± 0.00259 h^{-1} (0.9482)
	Zero-order[c]	
	Initial	0.356 ± 0.0097 $\mu g \cdot h^{-1}$ (0.9018)
	Second	1.086 ± 0.0393 $\mu g \cdot h^{-1}$ (0.9922)

[a]Standard error of the mean
[b]Coefficient of determination (r^2)
[c]Zero-order rate constant per mg of HAM

on the surface and/or entrapped in the near inner surface of HAMs and the slow release at the second phase is due to the release of the entrapped free MTX from the core of the HAMs and trace of MTX released from MTX-HSA conjugate on the surface of HAMs by hydrolysis.

Drug release by protease

Fig. 3 shows the *in vitro* release of MTX in the presence of protease. The release of MTX from the HAMs are quite different from that in the absence of protease. The MTX was released by first- or zero-order from the HAMs in the presence of protease, and protease accelerated the release of MTX from the HAMs. Approximately 100, 89.0, 75.0, 66.0 and 61.0% of MTX were released from HAMF, HAMCF 3, HAMCF, HAMC3F and HAMC, respectively for up to 24 h of incubation in the presence of protease and the corresponding values were 30.2, 19.0, 10.0, 6.5 and 2.2%, respectively in the absence of protease. The release of MTX from HAM decreased with increased entrapment of MTX-HSA conjugates in HAM; the time for 50% release ($t_{1/2}$) from HAMF, HAMCF3, HAMCF, HAMC3F and HAMC were approximately 2.2, 4.8, 11.4, 17.8 and 20 h, respectively.

For all sets of data from release test, percentages of MTX remained in HAMs (P) were plotted as a function of time according to the two relationships being considered, i.e. P versus time and log P versus time as reported[13], and the results are summarized in Fig. 3 and Table IV. Fig. 3 and Table IV demonstrate that the *in vitro* release of MTX in the presence of protease vary with the ratios of MTX-HSA conjugates to MTX; the data set from HAMF, HAMCF3 and HAMCF fits better to monophasic first-order profile more adequately than to zero-order profile, that of HAMC3F to monophasic zero-order, and that of HAMC to bi-phasic zero-order. The MTX was released fast from HAMF exponentially from the first, whereas the MTX in HAMC was released by bi-phasic zero order; the initial release rate constant up to approximate 4 h was 0.246± 0.0097 $\mu g \cdot h^{-1}$ (lag phase), much smaller than the second rate constant of 1.086± 0.0393 $\mu g \cdot h^{-1}$. It is to be noted that the release of MTX from HAMC3F and HAMC were fitted to monophasic and bi-phasic zero order kinetics, respectively. Above results suggested that zero-order release rate can be achieved by adjusting the ratio of MTX-HSA conjugates to MTX in the preparation of HAMs such as HAMC3F. Therefore, *in vivo* level of drug released from HAM, which is likely due to matrix degradation by lysosomal enzyme, might be controlled by varying the ratio of MTX-HSA conjugate to MTX.

CONCLUSIONS

On the basis of these results, if HAM containing free MTX and MTX-HSA conjugates is administrated, free MTX is released first from the HAM and thereafter MTX from MTX-HSA conjugates entrapped in HAM is released. It is suggested that *in vivo* drug release might be controlled rationally by encapsulating free MTX for loading dose and MTX-HSA conjugates for maintenance dose simultaneously within HAMs and the constant MTX concentrations at targeting tissue could be obtained over prolonged periods. However, this hypothesis needs to be validated. Furthermore, if HAM containing two synergistic drugs with schedule depen-

dency, for example free MTX and conjugated 5-FU may be administered for cancer chemotherapy, it expected that it may have imparted clinical implications. Therefore, this investigation suggests the potential of HAM containing simultaneously free drug and conjugated drug as a useful drug delivery system in cancer chemotherapy.

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