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PHARMACOKINETICS AND ORGAN-DISTRIBUTION OF ^{3}H-METHOTREXATE AND ^{3}H-METHOTREXATE-HUMAN SERUM ALBUMIN CONJUGATES IN MICE

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ABSTRACT

This investigation has been made to elucidate the pharmacokinetics and organ distribution of ^{3}H-methotrexate (MTX) and ^{3}H-MTX-human serum albumin (HSA) conjugate in mice and evaluate the feasibility to develop MTX-HSA conjugate as a useful anticancer delivery system. ^{3}H-MTX-HSA conjugate was synthesized by coupling of ^{3}H-MTX with HSA by carbodiimide reaction using EDC. ^{3}H-MTX (treatment I) and ^{3}H-MTX-HSA conjugates (treatment II) were injected intravenously via tail vein of ICR mice. At the designated time, blood was collected via heart puncture, and 4 mice were sacrificed. The liver, spleen, kidney, and lung were excised, and total radioactivity was measured. The mean plasma total radioactivity declined polyexponentially with a mean terminal half-life of 2.7 days from treatment I, however, the radioactivity declined rapidly for up to 3 days after the dose and decreased very slowly thereafter for up to 21 days after the dose. The mean AUQ in the liver, kidney, spleen, and lung was higher from treatment II than that from treatment I. It clearly indicated that ^{3}H-MTX-HSA conjugates were more uptaken into the organ than

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that of ^{3}H-MTX, and ^{3}H-MTX was released slowly from the conjugates. In treatment II, the weighted-average overall drug targeting efficiency(Te) for the liver was higher than in treatment I (86.4 vs 67.8%), and the weighted-average relative tissue exposure(Re) was 6.4 for the liver. It suggested that administration of ^{3}H-MTX-HSA conjugates might have good targeting ability to the liver.

INTRODUCTION

The ideal dosage form in cancer chemotherapy is "the one that provides a specific delivery of anticancer agents to the tumor site at a controlled rate over a long period of time without considerable interaction with normal tissues" (1). For this purpose, anticancer drug-macromolecule were synthesized (2-4), and their anticancer activities were reported (4-9). For example, MTX-bovine serum albumin (BSA) conjugates increased the survival time of mice bearing the ascitic form of L1210 (9, 10), and the conjugates were proved to be more effective than free MTX against subcutaneously transplanted Lewis lung carcinoma (5). Moreover, radioactive albumin is absorbed into the neoplastic cell (11), and albumin is uptaken actively into tumor cells by pinocytosis (12). Some of the MTX-rabbit serum albumin (RSA) conjugates seemed to be uptaken into tissues, and MTX was released slowly from the conjugates (13). The above results led us to study MTX-HSA conjugates as a means of increasing the therapeutic benefit (as a drug targeting agent) of MTX.

This work was undertaken to elucidate the tissue distribution and targeting efficiency of ^{3}H-MTX-HSA conjugates in mice over a long period and evaluate the feasibility to develop MTX-HSA conjugate as a useful anticancer delivery system.

EXPERIMENTAL METHODS

Chemicals

MTX was supplied by Choong-Wae Pharm. Co. (Seoul, Korea) and [3′,5′,7-^{3}H]- MTX (250 mCi/mmol, TRA224) was purchased from Amersham International (Buckinghamshire, U.K.). Human serum albumin (Fraction V) and 1-ethyl-3- (3-dimethylaminopropyl) carbodiimide hydrochloride (EDC, Protein sequencing reagents) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Sephadex® G-75-40 (particle size: 10-40 μm)

was purchased from Pharmacia Fine Chemicals (Uppsala, Sweden) and Soluene-350® (0.5N quaternary ammonium hydroxide in toluene) as tissue solubilizer was purchased from Packard Instrument Co. (Downers Grove, IL, U.S.A.). All other chemicals were of reagent grade and were used without further purification.

Animals

Male ICR mice, weighing 18-22 g, were purchased from Experimental Animal Breeding Center of Seoul National University (Seoul, Korea). Animals were fed commercial rodent chow (Samyang Co., Seoul, Korea) and water *ad libtum*.

Synthesis of ^{3}H-MTX-HSA Conjugate

^{3}H-MTX-HSA conjugate was synthesized by modifying the methods reported previously (2,4,14). To 100 mg of HSA dissolved in 5 ml of distilled water, was added MTX (20 mg) and 80 μCi of ^{3}H-MTX in 2 ml of 0.05 N NaOH, and 100 mg of EDC dissolved in 2 ml of 0.05 N HCl, respectively. After MTX solution was added to HSA solution, the pH of this solution was adjusted to 6.0 with 0.1 N HCl. EDC solution was slowly added to this solution at pH 5.0-6.0 for about 7 h. The reaction mixture was stirred for 12 h at 4 °C. Then, to the reaction mixture, EDC solution which contains 50 mg of EDC in 1.0 ml of 0.05 N HCl was slowly added for 4 h. The resultant solution was loaded on Sephadex® G-75-40 column (2.5 x 30 cm). The first conjugate fraction was discarded, the remaining macromolecular fractions were pooled and dialyzed (SPECTRAPOR® membrane tubing, m.w. cut off: 6,000-8,000, cylinder diameter: 14.6 mm, Spectrum Medical Ind.) against distilled water in the dark at 4 °C. The solution was filtered through membrane filter (0.45 μm) and then lyophilized.

Determination of MTX/HSA ratio in the Conjugates

The ratio of MTX to HSA in the conjugate was calculated from the absorbance at 370 nm or the radioactivity measured by liquid scintillation counter for determining MTX and the absorbance at 540 nm for measuring HSA concentration by the Biuret method (15).

Intravenous Study

The 150 nCi (dissolved in 0.2 ml of injectable 0.9% NaCl solution) of ^{3}H-MTX (treatment I), and ^{3}H-MTX-HSA conjugates (treatment II) were injected via tail vein of mice,

respectively. Blood sample was collected directly via heart puncture into heparinized syringe with appropriate time intervals and was immediately centrifuged to minimize the "blood storage effect" on the determination of total plasma radioactivity (16). After each blood sampling, 4 mice were sacrificed by cervical dislocation, and the lung, liver, spleen and kidney were removed, rinsed with cold normal saline, blotted dry, and weighed.

Analysis of total radioactivity

A portion of plasma and each organ were solubilized with 1 ml of Soluene 350® (0.5 N quaternary ammonium hydroxide in toluene, Packard Instrument Co.) per 0.2 g of organ in a counting vial. The vial was kept at 50 °C for 12 hr, and 0.2 ml of isopropyl alcohol and 0.4 ml of 30% hydrogen peroxide were added to minimize color quenching. In order to neutralize the solution, about 0.1 ml of 5 N HCl was added and then 10 ml of scintillation cocktail, 5 ml of Scinti-A® XF (Packard Instrument Co.) and 5 ml of PPO (2, 5-Diphenyloxazole, Sigma Chemical Co.)/ POPOP (1, 4-[2-(5-phenyloxazolyl)] benzene, Sigma Chemical Co.) cocktail were added.

The total radioactivity in the biological sample was determined by the liquid scintillation counter (Rack Beta, LKB-Wallac Co., Turku, Finland) after equilibration in the dark at 25 °C for at least 24 h prior to counting. The obtained counts were corrected with the standard channel ratio method.

Data Analysis

The areas under the total amount of radioactivity-time curves (AUQ, the product of AUC multiplied by the weight of tissue) were estimated by trapezoidal rule-extrapolation method (16). Two indices for the evaluation of drug delivery into the targeting site were calculated (17,18) based on AUQ; one is the weighted-average relative tissue exposure(Re), and the other is the weighted-average overall drug targeting efficiency (Te),

$$(Re)_i = \frac{(AUQ)_{i\,II}}{(AUQ)_{i\,I}} \qquad (1)$$

$$(Te)_i = \frac{(AUQ)_i}{\Sigma(AUQ)_i} \qquad (2)$$

where, i is the each organ, and I and II are the treatments I and II, respectively. Successful specific organ targeting of drug is indicated by Re value of greater than one for the targeting organ (i.e. liver), and less than 1 or close to 1 for the non-targeting organ.

Statistical analysis

The radioactivity remaining in each tissue at each observed time were analyzed for statistical significance ($p<0.05$ vs treatment I) by unpaired t-test between treatments I and II.

RESULTS AND DISCUSSION

Conjugation of ^{3}H-MTX-HSA

^{3}H-MTX-HSA conjugates were synthesized by the carbodiimide reaction (14). The carbodiimides activate free carboxyl groups by forming the corresponding O-acylisourea intermediates which can be further react and give symmetrical anhydrides. These intermediates can react with available nucleophiles such as amino groups so that amide bonds are formed between the drug and HSA. Since both MTX and HSA possess carboxyl and amino groups in their structures, it is assumed that the conjugates might be a heterogeneous products consisting of mixtures of albumin polymers with varying quantities of covalently bound drug. In the previous studies reported by Kim et al.(24) and Halbert et al.(4), the *in vitro* release from MTX-HSA or MTX-BSA conjugates was a biphasic process, these results suggested that the MTX should be attached to the albumin by two distinct types of linkage and MTX-HSA conjugates should be a heterogeneous compound. In the preliminary experiment, the yield of MTX-HSA conjugates reached a maximum value when 100 mg of EDC was added to the mixtures of 100 mg of HSA and 20 mg of MTX for 7 hrs. The molar ratio of MTX to HSA in the conjugates was 17.6, and similar value, 15.3 was reported (4) when 25.0 mg of EDC was added immediately to the mixture of 100 mg of BSA and 40 mg of MTX.

When EDC solution was rapidly added to the reaction mixture in the preparation of MTX-HSA conjugates, the condition of reaction was changed and the molecules of HSA could be cross-linked among themselves. Namely, as HSA has both carboxyl and amino groups, the molecules of HSA could be cross-linked or polymerized among themselves in the strong reactive conditions. The cross-linked components were pooled in the first

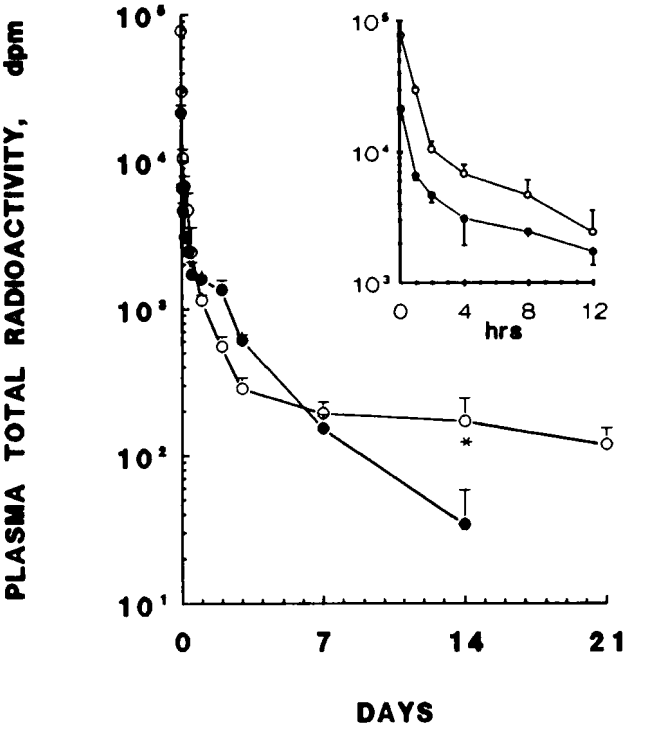

FIGURE 1

The mean total radioactivity-time curves after intravenous injection of ^{3}H-methotrexate (MTX, treatment I, ●) and ^{3}H-MTX-human serum albumin (HSA) conjugates (treatment II, ○) to mice.
The bars represent standard deviation. * : $p < 0.05$, ** : $p < 0.01$

several fractions of the product peak and discarded. The larger amount of free MTX is used in the synthesis, the higher the molar ratio of MTX to HSA in the conjugates can be accomplished but the less the yield of conjugated MTX may be. Hence, it is very significant to optimize the preparation method so that cross-linking between HSA polymers can be minimized and the yield of MTX-HSA conjugates can be maximized.

Evaluation of Drug Delivery

The mean total radioactivity in plasma from treatments I and II is shown in Fig 1. It is to be noted that in the present study, total radioactivity (such as the sum of radioactivity

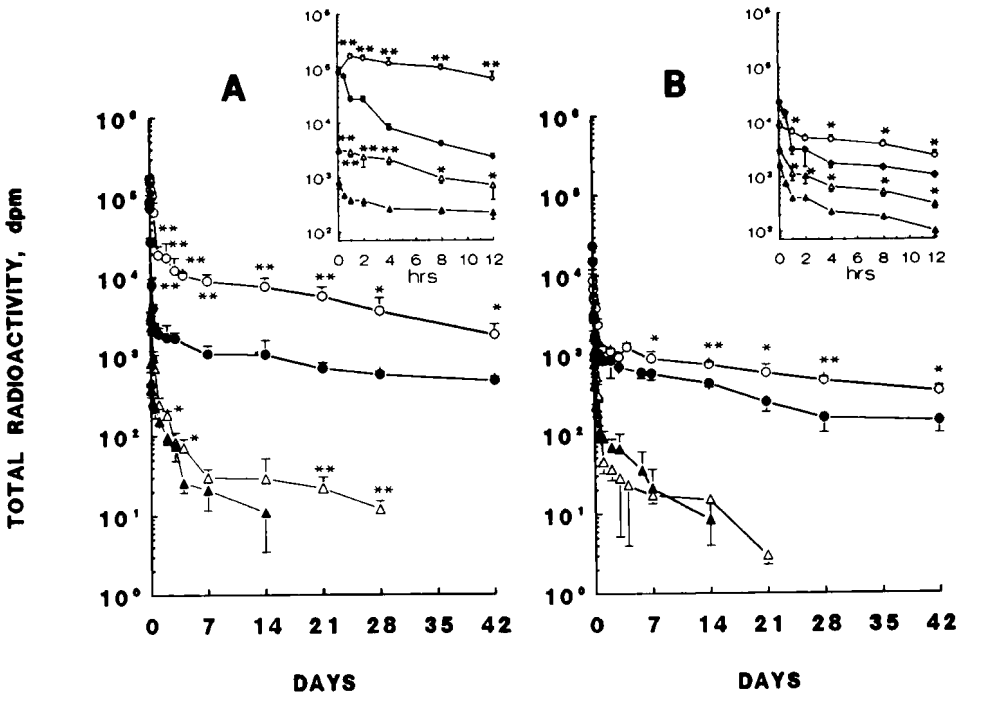

FIGURE 2

The mean total radioactivity-time curves in liver (Fig. A. ●, ○), spleen (Fig.A. ▲ , △), kidney (Fig. B. ●, ○), and lung (Fig. B. ▲ , △) after intravenous injection of ^{3}H-MTX (treatment I, closed symbols) and ^{3}H-MTX-HSA conjugates (treatment II, open symbols) to mice.
The bars represent standard deviation. * : $p < 0.05$, ** : $p < 0.01$

of ^{3}H-MTX, ^{3}H-MTX-HSA conjugates and their metabolites) was measured. Therefore, the total radioactivity does not represent the radioactivity of only ^{3}H-MTX or ^{3}H-MTX-HSA conjugates. The plasma total radioactivity declined polyexponentially with an apparent terminal half-life of 2.70 days from treatment I. However, the total radioactivity declined rapidly for up to 3 days after the dose and was almost constant thereafter for up to 21 days after the dose from treatment II. It was reported that though albumin is a large molecule, it is not confined to the intravascular space, and over 30% of total exchangeable albumin which is synthesized in the liver might be in extravascular space, such as in muscle and skin (19). Serum albumin has been demonstrated to accumulate

TABLE I. The area under the total amount radioactivity vs time curve (AUQ), weighted-average relative exposure (Re), and weighted-average overall drug targeting efficiency (Te) after intravenous injection of ^{3}H-MTX (Treatment I) and ^{3}H-MTX-HSA conjugate to the tail vein of ICR mice.

Tissue	Treatment I		Treatment II		Re
	AUQ(dpm·day)	$(Te)_i$%	AUQ(dpm·day)	$(Te)_i$%	
Liver	56,464	67.8%	360,098	86.4%	6.4
Kidney	18,278	21.9%	41,028	9.9%	2.2
Spleen	824	1.0%	2,270	0.5%	2.8
Lung	590	0.7%	779	0.2%	1.3
Plasma*	7,138	8.6%	12,487	3.0%	1.7
Total	83,294	100%	416,662	100%	5.0

* Total blood volumes were considered as 7.87 ml per 100 g of body weight of mice.

at tumor site (20). Some of the MTX-HSA conjugates seemed to be uptaken into tissues and the rest of them were presented in plasma, and MTX was released slowly from the conjugates when the conjugates were infused intravenously to rabbits (13). Therefore, it could be expected that some of the injected ^{3}H-MTX-HSA conjugates could be uptaken into tissues and the rest of them were presented in plasma. ^{3}H-MTX is excreted via kidney, the main elimination organ for MTX in humans (21), dogs (22) and rabbits (23), however, ^{3}H-MTX-HSA conjugates themselves could not be excreted via kidney. Therefore, slower elimination of plasma total radioactivity from 3 to 21 days after the dose from treatment II might be "mainly" due to slow release of ^{3}H-MTX from ^{3}H-MTX-HSA conjugates which are uptaken into the tissues and presented in plasma. The total radioactivity in the liver and spleen from treatments I and II is shown in Fig. 2A and that in the kidney and lung is shown in Fig. 2B. The total radioactivity was always higher from treatments II than that from treatment I in all the organs studied. However, the total

radioactivity in the lung from treatment II was not significantly different from that of treatment I from 24 h after the dose. In both treatments I and II, the largest amount was distributed into the liver and the amount of distribution decreased in the kidney, spleen and lung in the order named. The AUQ, Te and Re values for the liver, kidney, spleen, lung and plasma from treatments I and II listed in Table I. The AUQ values from treatment II was always higher than those from treatment I in all the organs studied. It clearly indicated that ^{3}H-MTX-HSA conjugates were more uptaken into the organs than that of ^{3}H-MTX, and ^{3}H-MTX was released slowly from the conjugates. The Re value for liver was 6.4, and the Te value for the liver was higher from treatment II than that from treatment I (86.4 vs 67.8%). However, Te values for the kidney, spleen, lung and plasma were lower from treatment II than those from treatment I, respectively (9.9 vs 21.9%, 0.5 vs 1.0%, 0.2 vs 0.7 % and 3.0 vs 8.6 %). It suggested that administration of ^{3}H-MTX-HSA conjugates might have good targeting ability to the liver and alleviate the systemic side effects or toxicities of MTX. Similar result was also found (13) that the amounts of MTX remaining in liver was 3.67 times higher when MTX-HSA conjugates were infused in 30-min to rabbits than those of MTX. Especially, MTX was significantly less concentrated on the kidney from treatment II than that from treatment I. Namely, Re value for kidney was 2.2, which is lower than those for the liver and spleen. Te value for kidney was less from treatment II than that from treatment I (9.9 vs 21.9%). It suggested that the administration of MTX-HSA conjugates might have lower side effects in kidney than those of MTX (treatment I).

The present study thus has demonstrated that MTX-HSA conjugates could be a useful drug delivery system, especially in liver cancer chemotherapy.

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REFERENCES

1. H. Sezaki and M. Hashida, *CRC Crit. Rev. Ther. Drug Carrier Syst.*, **1**, 1-38 (1984).
2. B.C.F. Chu and J.M. Whiteley, *Mol. Pharmacol.*, **13**, 80-88 (1977).
3. G.W. Halbert and A.T. Florence, *J. Pharm. Pharmcol.*, **41**, 222-226(1989) .

4. G.W. Halbert, A.T. Florence and J.F.G. Stuart, *J. Pharm. Pharmacol.*, **39**, 871-876 (1987).
5. B.C.F. Chu and J.M. Whiteley, *J. Natl. Cancer Inst.*, **62**, 79-81 (1979).
6. B.C.F. Chu and J.M. Whiteley, *Mol. Pharmacol.*, **17**, 382-387 (1980).
7. B.C.F. Chu and S.B. Howell, *J. Pharmacol. Exp. Ther.*, **219**(2), 389-393 (1981).
8. B.C.F. Chu and S.B. Howell, *Biochem. Pharmcol.*, **30**, 2545-2552 (1981).
9. S.A. Jacobs, M. D'Urso-Scott and J.R. Bertino, *Ann. N.Y. Acad. Sci.*, **186**, 284-286 (1971).
10. N.G.L. Harding, *Ann. N.Y. Acad. Sci.*, **186**, 270-283 (1971).
11. H. Bushi, E. Fujiwara and D.C. Firszt, *Cancer Res.*, **21**, 371-377 (1961).
12. H.J.-P. Ryser, *Laboratory Investigation,* **12**, 1009-1017 (1963).
13. E.J. Yoon, H.W. Chang, M.G. Lee, H.J. Lee, M.K. Park and C.K. Kim, *Int. J. Pharm.*, **67**, 177-184 (1991).
14. C.K. Kim and Y.K. Oh, *Int. J. Pharm.*, **47**, 163-169 (1988).
15. T.G. Cooper, "The Tools of Biochemistry," John Wiley & Sons, New York, 1977, pp.51-55.
16. M.G. Lee, C.Y. Lui, M.L. Chen and W.L. Chiou, *Int. J. Clin. Pharmacol. Ther. Toxicol.*, **22**, 530-537 (1984).
17. P.K. Gupta and C.T. Hung, *Int. J. Pharm.*, **56**, 217-226 (1989).
18. J.M. Gallo, P.K. Gupta, C.T. Hung and D.G. Perrier, *J. Pharm. Sci.*, **78**, 190-194 (1989).
19. W.J. Jusko and M. Gretch, *Drug Metab. Res.*, **5**, 43-140 (1976).
20. J.C. Cerrottini and H. Isliker, *Eur. J. Cancer*, **3**, 111-124 (1967).
21. D.D. Shen and D.L. Azarnoff, *Clin. Pharmcokin.*, **3**, 1-13 (1978).
22. C.Y. Lui, M.G. Lee and W.L. Chiou, *Cancer Res.*, **45**, 1545-1548 (1985).
23. M-L. Chen and W.L. Chiou, *J. Pharmacokin. Biopharm.*, **11**, 499-513 (1983).
24. C.K. Kim, M.G. Lee, M.K. Park, H.J. Lee and H.J. Kang, *Arch. Pharm. Res.*, **12**, 186-190 (1989).