ELSEVIER

International Journal of Pharmaceutics 132 (1996) 175–182

international journal of pharmaceutics

The enhancement of liver targetability of [^{3}H]methotrexate-galactosylated serum albumin conjugate in mice

Chong-Rae Lim[a], Kyoung-Hee Oh[a], Kyoung Mi Kim[a], Soon-Hong Yuk[b], Hai-Bang Lee[b], Chong-Kook Kim[a,*]

[a]*College of Pharmacy, Seoul National University, San 56-1, Shinlim-Dong, Kwanak-Gu, Seoul 151-742, South Korea*
[b]*KRICT, P.O. Box 107, Yusung, Taejeon 305-606, South Korea*

Received 24 August 1995; accepted 30 October 1995

Abstract

[^{3}H]Methotrexate-lactosaminated bovine serum albumin conjugates ([^{3}H]MTX-LBSA) were prepared and their organ distribution and pharmacokinetics were investigated in order to develop a liver-specific anticancer agent. Lactosaminated bovine serum albumin (LBSA) was synthesized by the reductive amination method. LBSA was then conjugated with [^{3}H]methotrexate ([^{3}H]MTX) through carbodiimide reaction to produce [^{3}H]MTX-LBSA. The liver targeting ability of [^{3}H]MTX-LBSA was evaluated by measuring the total radioactivity in each organ and plasma after i.v. administration via the tail vein of mice. The plasma level of [^{3}H]MTX-LBSA declined more rapidly than that of [^{3}H]MTX conjugated with ordinary bovine serum albumin ([^{3}H]MTX-BSA). The weighted-average overall drug targeting efficiency (T_e^*) for the liver of [^{3}H]MTX-LBSA was higher than that of [^{3}H]MTX-BSA (81 vs. 56%). The ratios of the area under the total amount of drug-time curve (AUQ) in the liver to that in the kidney (t_e^*) for [^{3}H]MTX-LBSA and [^{3}H]MTX-BSA were 12.4 and 8.8, respectively. All these suggested that MTX-LBSA conjugate is one of the advanced MTX delivery systems which can concentrate MTX in the liver.

Keywords: Galactosylated serum albumin; Liver targetability; Methotrexate; [^{3}H]Methotrexate-galactosylated serum albumin conjugate; [^{3}H]Methotrexate-serum albumin conjugate

1. Introduction

Ashwell and Morell (1974) have reported that the glycoprotein molecule was rapidly cleared from the blood circulation by removing the terminal sialic acid from native mammalian serum glycoproteins, thus exposing the galactose residue. The ligand and degradation products of these galactose terminal glycoproteins were ultimately found within the lysosomal fraction of hepatocytes (Gregoriadis et al., 1970). Further investigations revealed that more than 20 plasma proteins are cleared from the mammalian circulation via the hepatic receptor for galactose-terminal glycoproteins called a hepatic asialoglycoprotein recep-

* Corresponding author. Tel.: + 82 2 880 7867.

SSDI 0378-5173(95)04381-J

tor (Ashwell and Harford, 1982). The recognition of galactose-terminal glycoproteins via the asialoglycoprotein receptor provides a highly efficient system for targeting macromolecules to the hepatocyte (Atti et al., 1980; Fiume et al., 1981; Wu et al., 1983).

In this study, the mammalian serum glycoprotein of lactosaminated bovine serum albumin (LBSA) was synthesized as a drug carrier in order to constitute an effective drug delivery system for the treatment of hepatic malignancy. It was also reported that most of the particles such as liposomes, microspheres, etc. accumulate in the liver (Kim et al., 1993; Kim et al., 1994). However, the disposition of drug to the liver by the drug-galactosylated serum albumin conjugate system undergoes a different pathway from that by the microparticulate system. The drug is mainly taken up by the hepatocyte via the hepatic receptor for galactose-terminal glycoproteins in the former system, but the reticuloendothelial system (RES) in the liver has a major role in hepatic uptake in the latter system. Relatively larger particles are taken up by the Kupffer cells and smaller ones are taken up by both Kupffer cells and parenchymal cells (Sato et al., 1986). Also, the release mechanism of drug-lactosaminated albumin conjugate may differ from that of microparticulates. Most microparticulate-loading drugs such as liposomes and microcapsules release the drug at once after they break up, while the conjugates release the drug molecules gradually as they degrade.

Coupling the drug to a carrier, which is selectively taken up by the cells where the pharmacological action is required, is an important factor for an ideal use of the drug. Methotrexate (MTX), used as a model drug in this study, is a potent inhibitor of dihydrofolate reductase (Chabner et al., 1975; Bleyer, 1978), and many studies on MTX-common albumin conjugates were performed. It was reported that methotrexate-bovine serum albumin conjugates (MTX-BSA) increased the survival time of mice bearing the ascitic form of L1210 (Jacobs et al., 1971), and were more effective than free MTX against subcutaneously transplanted Lewis lung carcinoma (Chu and Whiteley, 1979). Some methotrexate-rabbit serum albumin conjugates appeared to be taken up into tissues, and MTX was released slowly from the conjugates after intravenous administration of the conjugates to rabbits (Yoon et al., 1991). It was also found that [^{3}H]methotrexate-bovine serum albumin conjugates ([^{3}H]MTX-BSA) were more disposed to the organ than free [^{3}H]MTX (Kim and Hwang, 1993). However, few studies have been reported for galactose-terminal albumin conjugates.

In this laboratory, lactosaminated bovine serum albumin (LBSA) was synthesized and conjugated with [^{3}H]methotrexate ([^{3}H]MTX) to prepare [^{3}H]methotrexate-lactosaminated bovine serum albumin conjugate ([^{3}H]MTX-LBSA) for the development of a liver targeting drug delivery system. The pharmacokinetics and organ-targeting ability of this conjugate were evaluated after i.v. injection via the tail vein of mice and compared with those of [^{3}H]MTX-BSA.

2. Materials and methods

2.1. Materials

MTX was kindly supplied by Choong-Wae Pharm. Co. (Seoul, South Korea) and [L-Glutamyl 3,4-^{3}H]methotrexate ([^{3}H]MTX, 41 Ci/mmol, NET-730) was purchased from Amersham International Plc. (Buckinghamshire, UK). Bovine serum albumin (BSA, Fraction V) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) were purchased from Sigma Chemical Co. (St. Louis, MO). Lactose monohydrate and hydrogen peroxide were obtained from Junsei Chemical Co. (Tokyo, Japan). Sephadex® G-75-40 (particle size: 10–40 μm) was a product of Pharmacia Fine Chemicals (Uppsala, Sweden). Scinti-A® XF scintillation cocktail and Soluene-350® (0.5 N quaternary ammonium hydroxide in toluene) were purchased from Packard Instrument Co. (A Canberra Co., Downers Grove, IL). PPO/POPOP cocktail was prepared using 2,5-diphenyloxazole (PPO, Sigma Chemical Co.), 1,4-[2-(5-phenyloxazolyl)]benzene (POPOP, Sigma Chemical Co.), and Triton® X-100 (Duksan Pharmaceutical Co., Seoul, South Korea). All other chemicals were of reagent grade and used as received.

2.2. Animals

Male ICR mice, weighing 20–30 g, were purchased from Experimental Animal Breeding Center of Seoul National University (Seoul, South Korea). Animals were fed with commercial rodent chow (Samyang Co., Seoul, South Korea) and water ad libitum.

2.3. Synthesis of [^{3}H]MTX-LBSA conjugate

LBSA was synthesized by modifying the reductive amination method reported previously (Schwartz and Gray, 1977) as shown in Fig. 1. One gram of α-lactose and 1 g of sodium cyanoborohydride ($NaCNBH_3$) were added to 680 mg of BSA dissolved in 50 ml of 0.2 M potassium phosphate buffer (pH 8.0). This reaction mixture was stirred at 37°C for 260 h, and the LBSA fraction was isolated. The LBSA fraction was then concentrated by ultrafiltration and lyophilized. The ratio of galactose to BSA in the LBSA was calculated by measuring galactose concentration from the absorbance at 490 nm using phenol/sulphuric acid method (Dubios et al., 1956), and BSA concentration from the absorbance at 660 nm using the modified Lowry method (Lowry et al., 1951). It was found that the amount of galactose attached to BSA in LBSA was affected by the quantities of reactants (α-lactose, BSA and sodium cyanoborohydride), reaction time and stirring rate. In the present study, 18–20 mol of galactose was incorporated into BSA, values similar to those reported by Fiume et al. (1982).

[^{3}H]MTX-LBSA conjugate was synthesized using methods reported previously (Chu and Whiteley, 1977; Halbert et al., 1987; Kim and Oh, 1988). The mixture of MTX (5 mg) and [^{3}H]MTX (96.2 μCi) dissolved in 1 ml 0.05 N NaOH was added to 30 mg of LBSA dissolved in 1.2 ml of distilled water, and the pH of the solution was adjusted to 6.0 with 0.1 N HCl. To this solution, 30 mg of EDC dissolved in 0.6 ml of 0.05 N HCl was added slowly for 4 h. The reaction mixture was kept for 12 h at 4°C with stirring. Ten milligrams of EDC dissolved in 0.2 ml of 0.05 N HCl was added for 2 h to complete the reaction. The resultant solution was loaded on a Sephadex G-75-40 column, and the first conjugate fraction was discarded. The remaining macromolecular conjugate fraction was dialyzed using a semipermeable membrane (Spectrapor®, Spectrum Medical Ind., Houston, TX), and then lyophilized. The molar ratio of MTX to LBSA in the conjugate was calculated from the radioactivity measured by liquid scintillation counter (Rack Beta, LKB-Wallac Co., Turku, Finland), and found to be 10.2.

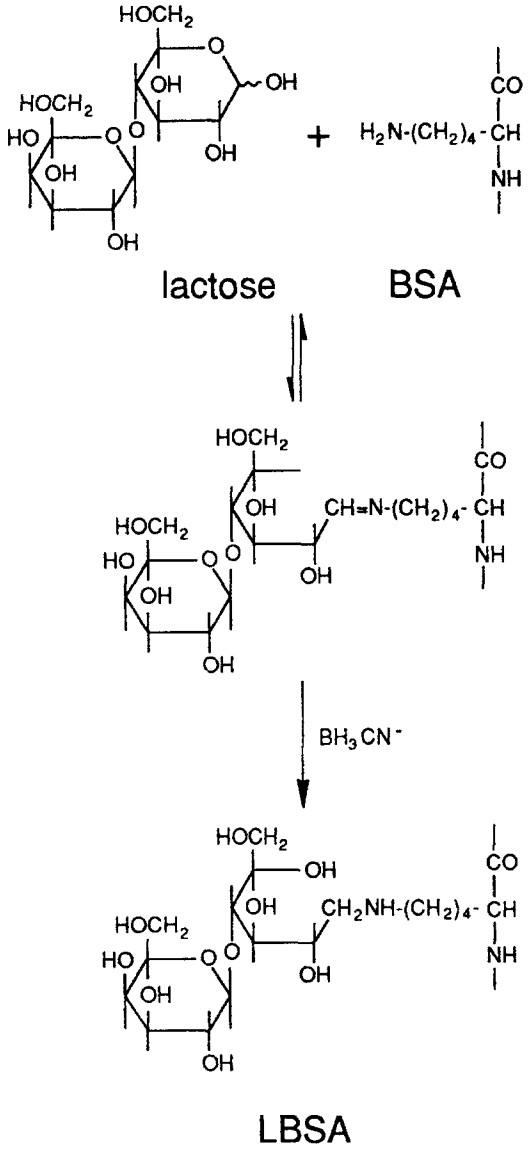

Fig. 1. Synthesis of LBSA by the reductive amination method.

[^{3}H]MTX-BSA conjugate was prepared by the same method as for the synthesis of [^{3}H]MTX-LBSA conjugate, and the molar ratio of MTX to BSA in the conjugate was determined by the above method.

2.4. Intravenous injection studies

[^{3}H]MTX-LBSA conjugate (150 nCi) or [^{3}H]MTX-BSA conjugate dissolved in 0.2 ml of injectable 0.9% NaCl solution was injected via the tail vein of mice. Blood samples were collected via heart puncture into heparinized syringes at each designated time and were immediately centrifuged. After blood sampling, mice were killed by cervical dislocation, and the lung, liver, spleen and kidney were removed, rinsed with cold normal saline, blotted dry with a paper towel and weighed.

2.5. Analysis of total radioactivity

In order to examine the total uptake of drug, drug-conjugates and all metabolites, radioactivity analysis was employed instead of using the HPLC method which requires the deproteinizing process, thus measures only the pure drug or each metabolite. In a counting vial, 0.2 g of each organ or plasma was solubilized with 1 ml of Soluene-350® (0.5 N quaternary ammonium hydroxide in toluene). The vial was kept at 50°C for 12 h, and then 0.2 ml of isopropyl alcohol and 0.4 ml of 30% hydrogen peroxide were added to minimize the color quenching. After neutralizing the mixture with 5 N HCl, 10 ml of scintillation cocktail (Scinti-A® XF:PPO/POPOP cocktail = 1:1) was added and equilibrated in the dark at 25°C for 24 h. The total radioactivity in the biological sample was determined by liquid scintillation counter (Rack Beta, LKB-Wallac Co., Turku, Finland). Total radioactivity was obtained using the standard channel ratio quenching correction method. The mean concentration of MTX in plasma was calculated from the total radioactivity representing the radioactivity of [^{3}H]MTX, conjugates ([^{3}H]MTX-LBSA or [^{3}H]MTX-BSA) and their metabolites.

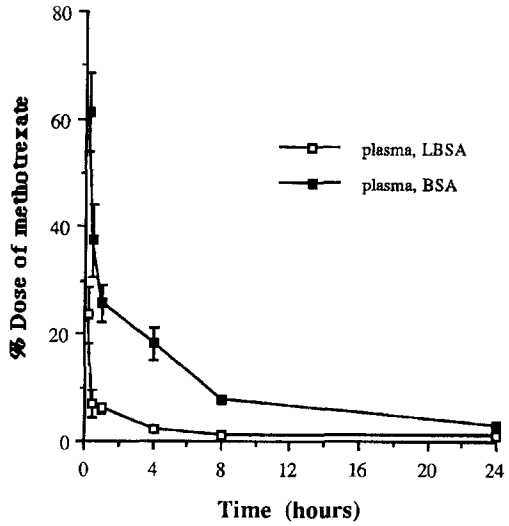

Fig. 2. The plasma percent doses of MTX after intravenous injection of [^{3}H]MTX-LBSA and [^{3}H]MTX-BSA. Bars represent the standard deviation.

2.6. Analysis of data

The areas under the total amount of radioactivity-time curves (AUQ, the product of AUC multiplied by the weight of tissue) were estimated by the trapezoidal rule-extrapolation method (Chiou, 1978). The weighted-average drug targeting efficiency against a given non-targeting tissue (t_e^*) and the weighted-average overall drug targeting efficiency (T_e^*) were calculated based on AUQ (Gupta and Hung, 1989) as follows;

$$(T_e^*)_i = (\mathrm{AUQ})_i / \sum \mathrm{AUQ} \quad (1)$$

$$t_e^* = \mathrm{AUQ}_{\mathrm{liver}} / \mathrm{AUQ}_j \quad (2)$$

where i denotes each organ and j represents any non-targeting organ.

Levels of statistical significance were assessed using the unpaired t-test between the two means for unpaired data. Significant differences were judged as a P-value of less than 0.05.

3. Results and discussion

In Fig. 2, the mean percent doses of MTX in plasma are plotted with time for [^{3}H]MTX-LBSA and [^{3}H]MTX-BSA. After intravenous injection, the plasma concentration of MTX declined poly-exponentially in both [^{3}H]MTX-LBSA and [^{3}H]MTX-BSA. The percent dose of MTX in plasma from [^{3}H]MTX-LBSA was significantly lower and decreased more rapidly than that from [^{3}H]MTX-BSA after the injection. In the previous study, it was reported that some of the MTX-human serum albumin conjugates were taken up into tissues and the rest were present in plasma after intravenous infusion to rabbits (Yoon et al., 1991). Kim and Hwang (1993) demonstrated that more human serum albumin conjugates were taken up into the organ than free MTX, and released MTX slowly from the conjugates. Therefore, the rapid elimination of the plasma percent dose of MTX from [^{3}H]MTX-LBSA might be due to the higher tissue disposition of [^{3}H]MTX-LBSA compared to that of [^{3}H]MTX-BSA. Since [^{3}H]MTX-LBSA includes an exposed galactose residue in its molecular structure, it is considered that MTX from this conjugate would be transferred especially to the liver via the asialoglyco-

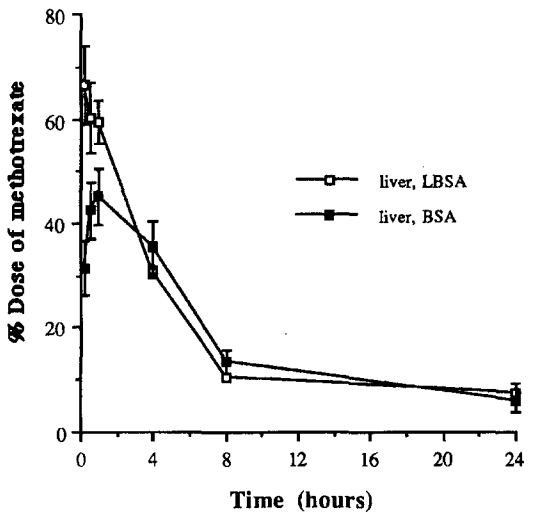

Fig. 3. The liver percent doses of MTX after intravenous injection of [^{3}H]MTX-LBSA and [^{3}H]MTX-BSA. Bars represent the standard deviation.

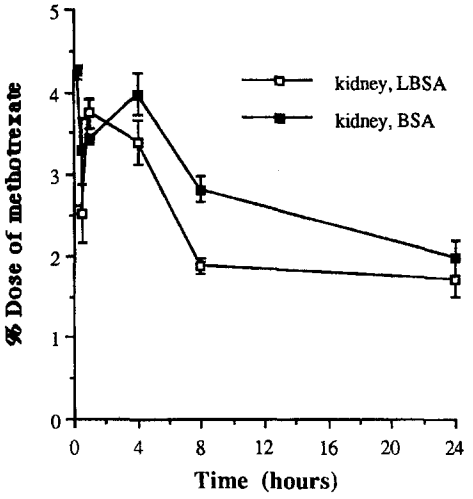

Fig. 4. The kidney percent doses of MTX after intravenous injection of [^{3}H]MTX-LBSA and [^{3}H]MTX-BSA. Bars represent the standard deviation.

protein receptor in the hepatic cell (Ashwell and Harford, 1982). As shown in Figs. 2 and 3, it can be seen that [^{3}H]MTX-LBSA was rapidly cleared from the plasma and was considerably taken up into the liver immediately after the injection, while [^{3}H]MTX-BSA was slowly taken up.

Because MTX is known to cause toxicity mainly in kidney and GI tract (Shen and Azarnoff, 1978; Evans et al., 1986), the percent dose of MTX in kidney from the conjugates was investigated and plotted in Fig. 4. The total percent dose distributed into kidney was significantly lower compared with that distributed into liver or plasma. Generally, the total percent dose was higher from [^{3}H]MTX-BSA than from [^{3}H]MTX-LBSA. In the previous study (Kim and Hwang, 1993), MTX was less concentrated in the kidney from MTX-albumin conjugates than that from free MTX. Specific assays by the HPLC method showed that the percent dose of parent drug, excluding MTX metabolites and MTX conjugates, in kidney 2 h after the intravenous injection of free MTX, MTX-BSA and MTX-LBSA was 4.19, 0.86 and 0.44, respectively (Han, 1994). Also, a similar trend was observed for the concentration of 7-OH-MTX, a main metabolite of MTX, by the same assay (Han, 1994). Therefore, it can be seen that MTX-galactosylated albumin

Table 1
The tissue distribution of [^{3}H]MTX (mean % dose ± S.D.) after intravenous injection of [^{3}H]MTX-LBSA and [^{3}H]MTX-BSA

Time	Liver	Kidney	Spleen	Lung	Heart
[^{3}H]MTX-LBSA					
15 min	66.6 ± 7.48	2.57 ± 0.051	0.483 ± 0.090	0.508 ± 0.107	0.205 ± 0.026
30 min	60.0 ± 6.72	2.52 ± 0.357	0.395 ± 0.044	0.416 ± 0.028	0.180 ± 0.015
1 h	59.3 ± 4.12	3.74 ± 0.170	0.382 ± 0.006	0.617 ± 0.060	0.190 ± 0.025
4 h	30.6 ± 1.06	3.39 ± 0.269	0.151 ± 0.033	0.325 ± 0.094	0.155 ± 0.035
8 h	10.4 ± 0.561	1.87 ± 0.104	0.106 ± 0.005	0.110 ± 0.025	0.048 ± 0.014
1 day	7.48 ± 2.00	1.70 ± 0.219	0.227 ± 0.074	0.119 ± 0.048	0.024 ± 0.007
3 days	6.63 ± 1.55	1.24 ± 0.196	0.132 ± 0.026	0.131 ± 0.036	0.048 ± 0.009
[^{3}H]MTX-BSA					
15 min	31.4 ± 5.17	4.25 ± 0.081	1.06 ± 0.093	1.12 ± 0.017	0.245 ± 0.017
30 min	42.5 ± 5.36	3.28 ± 0.399	0.823 ± 0.123	1.05 ± 0.135	0.225 ± 0.019
1 h	45.0 ± 5.30	3.42 ± 0.058	0.229 ± 0.044	0.437 ± 0.038	0.187 ± 0.012
4 h	35.3 ± 5.01	3.98 ± 0.258	0.289 ± 0.094	0.462 ± 0.077	0.161 ± 0.020
8 h	13.2 ± 2.24	2.82 ± 0.153	0.168 ± 0.004	0.166 ± 0.044	0.055 ± 0.004
1 day	6.06 ± 2.28	1.97 ± 0.211	0.202 ± 0.054	0.139 ± 0.012	0.071 ± 0.023
3 days	5.78 ± 1.31	1.78 ± 0.278	0.141 ± 0.013	0.148 ± 0.015	0.092 ± 0.018

conjugate has minimum renal toxicity among free MTX, MTX-BSA and MTX-LBSA.

In Table 1, the tissue distributions of MTX (mean percent dose ± S.D.) for up to 3 days after the intravenous injection of [^{3}H]MTX-LBSA and [^{3}H]MTX-BSA are summarized. The mean percent dose of [^{3}H]MTX from [^{3}H]MTX-LBSA in the liver was about twice as high as that from [^{3}H]MTX-BSA (66.6 vs. 31.4) 15 min after the injection. After 1 h, the mean percent doses of [^{3}H]MTX from [^{3}H]MTX-LBSA and [^{3}H]MTX-BSA in the liver were 59.3 and 45.0, respectively. This is due to the rapid clearance of the galactose-terminal glycoprotein of [^{3}H]MTX-LBSA from the circulation and the high uptake to the liver through the asialoglycoprotein receptor (Ashwell and Harford, 1982). After 4 h, the amount of [^{3}H]MTX-LBSA remaining in the liver was lower than that of [^{3}H]MTX-BSA because of the fast metabolism and clearance in the liver after the rapid initial uptake.

Table 2
The $AUQ_{0->8h}$ and T_e^* of [^{3}H]MTX after intravenous injection of [^{3}H]MTX-LBSA and [^{3}H]MTX-BSA

Organ	[^{3}H]MTX-LBSA AUQ (T_e^*)	[^{3}H]MTX-BSA AUQ (T_e^*)
Liver	268 (81%)	242 (56%)
Kidney	21.6 (6.5%)	27.4 (6.4%)
Spleen	1.76 (0.53%)	2.72 (0.63%)
Lung	2.60 (0.79%)	3.66 (0.85%)
Heart	1.12 (0.34%)	1.17 (0.27%)
Plasma[a]	36.1 (11%)	152 (35.4%)
Total	331 (100%)	429 (100%)
t_e^{*b}	12.4	8.83

[a]Total blood volumes were considered as 7.87 ml per 100 g body weight of mice.
[b] AUQ_{liver}/AUQ_{kidney}.

In the kidney, the mean percent dose of [^{3}H]MTX from [^{3}H]MTX-LBSA is generally lower than that from [^{3}H]MTX-BSA. As shown in Table 1, the mean percent dose from [^{3}H]MTX-LBSA increased slowly for up to 1 h, then decreased rapidly. On the other hand, the mean percent dose of [^{3}H]MTX from [^{3}H]MTX-BSA shows a rapid increase within 15 min and then decreased slowly. The uptake of [^{3}H]MTX by the kidney within 15 min after the injection was 2.57 and 4.25% dose for [^{3}H]MTX-LBSA and [^{3}H]MTX-BSA, respectively, but the overall total amounts were similar.

The mean percent dose of [^{3}H]MTX in the other tissues is significantly lower when compared with that in the liver and kidney. The amounts of [^{3}H]MTX-LBSA in the spleen, lung and heart

were generally lower than those of [^{3}H]MTX-BSA as summarized in Table 1. Among these tissues, the lung uptakes both conjugates. The maximum value from [^{3}H]MTX-LBSA is 0.617% dose in the lung at 1 h, and that from [^{3}H]MTX-BSA is 1.12% dose in the same tissue at 15 min.

The areas under the total amount of radioactivity-time curves ($AUQ_{0->8h}$) for the liver, kidney, spleen, lung, heart and plasma are listed in Table 2. The weighted-average overall drug targeting efficiency (T_e^*) for the liver of [^{3}H]MTX-LBSA was significantly higher than that of [^{3}H]MTX-BSA (81 vs. 56%). The T_e^* for the kidney was not much different for both conjugates, but the ratios of AUQ in the targeting tissue of the liver to that in the non-targeting tissue of the kidney (t_e^*) for [^{3}H]MTX-LBSA was about 40% higher than that for [^{3}H]MTX-BSA (12.4 vs. 8.83).

4. Conclusion

Compared with [^{3}H]MTX-BSA, [^{3}H]MTX-LBSA was more rapidly cleared from the blood circulation and found to have the higher liver uptake and lower kidney distribution. The present study provides evidence that anticancer drugs from methotrexate can be selectively delivered into the hepatocyte by conjugation with the galactose-terminal glycoprotein of galactosylated serum albumin. The MTX-LBSA conjugate can thus be considered one of the advanced liver targeting delivery systems which can deliver more MTX to the liver and less to the kidney compared with MTX-BSA or free MTX (Kim and Hwang, 1993).

Acknowledgment

This work was supported in part by a research grant from HAN (G-7) project of the Ministry of Science and Technology.

References

Ashwell, G. and Harford, J., Carbohydrate receptors of the liver. *Annu. Rev. Biochem.*, 51 (1982) 531–534.

Ashwell, G. and Morell, A., The role of surface carbohydrates in the hepatic recognition and transport of circulating glycoproteins. *Adv. Enzymol.*, 41 (1974) 99–128.

Atti, A.D., Pittman, R.C. and Steinberg, D., Metabolism of native and lactosylated human low density lipoprotein: evidence for two pathways for catabolism of exogenous proteins in rat hepatocytes. *Proc. Natl. Acad. Sci. USA*, 77 (1980) 5923–5927.

Bleyer, W.A., The clinical pharmacology of methotrexate. *Cancer*, 41 (1978) 36–51.

Chabner, B.A., Mayer, C.E., Coleman, C.N. and Johns, D.G., The clinical pharmacology of antineoplastic agents. *New Engl. J. Med.*, 292 (1975) 1107–1113.

Chiou, W.L., Critical evaluation of potential error in pharmacokinetic studies using the linear trapezoidal rule method for the calculation of the area under the plasma level-time curve. *J. Pharmacokinet. Biopharm.*, 6 (1978) 539–546.

Chu, B.C.F. and Whiteley, J.M., High molecular weight derivatives of methotrexate as chemotherapeutic agents. *Mol. Pharmacol.*, 13 (1977) 80–88.

Chu, B.C.F. and Whiteley, J.M., Control of solid tumor metastases with high-molecular-weight derivatives of methotrexate. *J. Natl. Cancer Inst.*, 62 (1979) 79–81.

Dubios, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A. and Smith F., Colorimetric method for determination of sugars and related substances. *Anal. Chem.*, 28 (1956) 350–356.

Evans, W.E., Crom, W.R. and Yalowich, J., Applied therapeutics. In Evans, W.E., Schentag, J.J. and Jusko, W.J. (Eds.), *Applied Pharmacokinetics, Principles of Therapeutic Drug Monitoring*, 2nd edn., Spokane, WA. 1986, pp. 1009–1056.

Fiume, L., Busi, C., Mattioli, A., Balboni, P.G. and Barbanti-Brodno, G., Hepatocytes targeting of adenine-9-β-D-arabinofuranoside 5′-monophosphate (Ara-AMP) coupled to lactosaminated albumin. *FEBS Lett.*, 129 (1981) 261–264.

Fiume, L., Busi, C. and Mattioli, A., Lactosaminated human serum albumin as hepatotropic drug carrier: rate of uptake by mouse liver. *FEBS Lett.*, 146 (1982) 42–46.

Gregoriadis, G., Morell, A.G., Sternlieb, I. and Schenberg, I.H., Catabolism of desialylated ceruloplasmin in liver. *J. Biol. Chem.*, 245 (1970) 5833–5834.

Gupta, P.K. and Hung, C.T., Quantitative evaluation of targeted drug delivery systems. *Int. J. Pharm.*, 56 (1989) 217–226.

Halbert, G.W., Florence, A.T. and Stuart, J.F.B., Characterization of in-vitro drug release and biological activity of methotrexate-bovine serum albumin conjugates. *J. Pharm. Pharmacol.*, 39 (1987) 871–876.

Han, J.-H., Development of liver targeting methotrexate delivery system using neo-galactosylated bovine serum albumin conjugate. Ph.D. dissertation, Seoul National University, Seoul, 1994.

Jacobs, S.A., D'Urso-Scott, M. and Bertino, J.R., Some biochemical and pharmacologic properties of amethopterin-albumin. *Ann. New York Acad. Sci.*, 186 (1971) 284–286.

Kim, C.-K. and Hwang, S.-J., Pharmacokinetics and organ-distribution of ^{3}H-methotrexate and ^{3}H-methotrexate-hu-

man serum albumin conjugates in mice. *Drug Dev. Ind. Pharm.*, 19 (1993) 961–970.

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.

Kim, C.-K., Hwang, S.-J. and Lee, M.G., The organ targetability of small and large albumin microspheres containing free and HSA conjugated methotrexate. *Int. J. Pharm.*, 89 (1993) 91–102.

Kim, C.-K., Lee, M.-K., Han, J.-H. and Lee, B.-J., Pharmacokinetics and tissue distribution of methotrexate after intraveous injection of differently charged liposome-entrapped methotrexate to rats. *Int. J. Pharm.*, 108 (1994) 21–29.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J., Protein measurement with folin phenol reagent. *J. Biol. Chem.*, 193 (1951) 265–275.

Sato, Y., Kiwada, H. and Kato, Y., Effects of dose and vesicle size on the pharmacokinetics of liposomes. *Chem. Pharm. Bull.*, 34 (1986) 4244–4252.

Schwartz, B.A. and Gray, G.R., Proteins containing reductive aminated disaccharide: synthesis and chemical characterization. *Arch Biochem. Biophys.*, 181 (1977) 542–549.

Shen, D.D. and Azarnoff, D.L., Clinical pharmacokinetics of methotrexate. *Clin. Pharmacokinet.*, 3 (1978) 1–13.

Yoon, E.J., Chang, H.W., Lee, M.G., Lee, H.J., Park, M.K. and Kim, C.-K., Pharmacokinetics of methotrexate after intravenous infusion of methotrexate-rabbit serum albumin conjugate to rabbits. *Int. J. Pharm.*, 67 (1991) 177–184.

Wu, G.Y., Wu, C.H. and Stochert, R.J., A model for specific rescue of normal hepatocytes during methotrexate treatment of hepatic malignancy. *Proc. Natl. Acad. Sci. USA*, 80 (1983) 3078–3081.