ELSEVIER

International Journal of Pharmaceutics 188 (1999) 39–47

international journal of pharmaceutics

www.elsevier.com/locate/ijpharm

Enhanced hepatocyte uptake and liver targeting of methotrexate using galactosylated albumin as a carrier

Jung-Hee Han [a], Yu-Kyoung Oh [a], Dong-Sup Kim [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] *National Institute of Toxicological Research, Korea Food and Drug Administration, San 5, Nokbun-Dong, Eunpyung-Gu, Seoul 122-704, South Korea*

Received 17 February 1999; received in revised form 5 May 1999; accepted 8 June 1999

Abstract

Liver targeting of drugs has wide therapeutic implications due to numerous liver-related diseases. Using conjugates of methotrexate (MTX) to variously galactosylated bovine serum albumin (BSA), we studied whether we could enhance the liver targeting of MTX, a model drug, via galactose receptors selectively abundant on the hepatocytes. Here, we report that the galactosylation of the carrier protein BSA significantly enhanced the hepatocyte uptake and liver targetability of MTX. In vitro, the amount of MTX taken up by rat hepatocytes was positively correlated with the galactose content in BSA. MTX conjugates were relatively stable in plasma, but released MTX with time in liver homogenates. These results imply that the conjugates would exert low toxicity in the blood, but have therapeutic activity in the liver by liberating MTX. In vivo, MTX-galactosylated BSA conjugates (MTX–L_{24}BSA) showed significantly different pharmacokinetics from free MTX or MTX–BSA conjugates. The plasma level of free MTX rapidly declined in a biexponential fashion with an apparent terminal half-life of 0.35 h. MTX–BSA conjugates showed the slowest decline with an apparent terminal half-life of 6 h, whereas MTX–L_{24}BSA showed a biphasic pattern; a rapid distributive phase with a half-life of 0.567 h and a slow terminal phase. MTX–L_{24}BSA showed the highest liver targetability, when evaluated in terms of two indices based on the area under the total amount of radioactivity–time curve (AUQ); Te^*(liver), % AUQ_{liver} to total AUQ, and te^*, the ratio of AUQ_{liver} to AUQ_{kidney}. Compared with free MTX and MTX–BSA, MTX–L_{24}BSA showed about twofold higher Te^*(liver) of 87.5%. The te^* of MTX–L_{24}BSA was 25- and fourfold higher than those of free MTX and MTX–BSA, respectively. Moreover, MTX–L_{24}BSA showed a gradual increase in the therapeutically active intact form of MTX in the liver while showing the lowest level of intact MTX in the kidney. These results suggest that galactosylated BSA has a great potential as an hepatocyte-directed and more effective liver targeting carrier of drugs for liver diseases. © 1999 Elsevier Science B.V. All rights reserved.

Keywords: Methotrexate; Methotrexate–galactosylated albumin conjugates; Pharmacokinetics; Tissue distribution; Liver targeting; Drug carrier

* Corresponding author.

0378-5173/99/$ - see front matter © 1999 Elsevier Science B.V. All rights reserved.
PII: S0378-5173(99)00206-9

1. Introduction

Chemotherapy using highly potent drugs such as anticancer agents has been restricted due to the lack of selectivity toward target sites. If the distribution of chemotherapeutic agents is directed to specific sites, it could dramatically enhance their therapeutic efficacy while decreasing toxic side effects at other sites of the body. In this respect, the liver has been one of the most desirable target organs in the body due to various liver-related metabolic, infectious diseases, and primary and metastatic cancers (Johnson, 1993; Rettinger et al., 1994).

Numerous efforts have been made to achieve liver targeting of chemotherapeutic agents. One approach is based on the active phagocytosis of the reticuloendothelial system in the liver by delivering drugs in particular carriers such as liposomes (Kim et al., 1994; Kim and Han, 1995) and microspheres (Kim et al., 1993; Anderson et al., 1994). Another approach is to use the receptor-mediated endocytosis by attaching drugs to carrier molecules that can interact with liver-associated receptors (Seymour et al., 1991; Sett et al., 1993). The major advantage of this approach is that one may design a more defined cellular level of drug targeting via cell type-specific surface receptors.

Of liver-associated surface receptors, the asialoglycoprotein receptor (galactose receptor) is known to be present only on the hepatocytes (Ashwell and Harford, 1982) with high density of 500 000 receptors per cell (Schwartz et al., 1980) and retained on several human hepatoma cell lines (Fallon and Schwartz, 1988). In addition, once a ligand binds to the galactose receptor, the ligand-receptor complex is rapidly internalized and the receptor recycles back to the surface (Ciechanover et al., 1983), which would allow the high binding capacity and efficient cellular uptake of galactosylated ligands. Thus, designing drug delivery systems for galactose receptor-mediated endocytosis would be useful for achieving hepatocyte/liver targeting, and further developing hepatoma cell targeting of chemotherapeutic agents.

Methotrexate has been widely used for the treatment of various neoplastic diseases including hepatoma and other liver disease such as cirrhosis (Tucker, 1992). Currently, chemotherapy using methotrexate is limited due to its toxic effects on the kidney and intestinal mucosa (Shen and Azarnoff, 1978). It has been reported that albumin conjugates of methotrexate (MTX) could alter the pharmacokinetics and tissue distribution of MTX (Kim and Hwang, 1993; Sett et al., 1993).

In the present study, to achieve more effective liver targeting of drugs via galactose receptors, galactosylated albumin and MTX were used as a carrier and model drug, respectively. Galactosylated albumin was chosen as a carrier since it is known to be non-immunogenic (Fiume et al., 1982). Using the covalent conjugates of MTX-galactosylated albumin, we tested whether we could modulate the tissue distribution and hepatocyte uptake of MTX. It was observed that MTX–galactosylated albumin conjugates not only enhanced the hepatocyte uptake and liver targetability of MTX with minimal distribution to the kidney, but also provided a higher level of intact MTX in the liver over a prolonged period.

2. Materials and methods

2.1. Materials

Lactose monohydrate was purchased from Junsei (Tokyo, Japan). Bovine serum albumin (BSA, fraction V), 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) and sodium cyanoborohydride were from Sigma (St Louis, MO). [^{3}H]MTX ([L-glutamyl-3,4-^{3}H]MTX) was from Dupont deNemours (Wilmington, DE). Lumasolve® (quaternary ammonium hydroxide), a tissue solubilizer, was from Lumac (Olen, Belgium). Scinti-A® XF scintillation cocktail and Soluene-350® (0.5 N quaternary ammonium hydroxide in toluene) were from Packard Instrument (Downers Grove, IL). MTX was kindly provided by Choong Wae Pharmaceutical (Seoul, South Korea). All other chemicals were of reagent grade and used without further purification.

2.2. Synthesis of galactosylated albumin–MTX conjugates

2.2.1. Synthesis of galactosylated BSA

Galactosylated BSA was synthesized by coupling lactose to the lysine residues of BSA via reductive amination of sodium cyanoborohydride (Schwartz and Gray, 1977). In brief, the mixture of 200 μM BSA, 23.4 mM lactose and 127.2 mM sodium cyanoborohydride in 0.2 M phosphate buffer (PB, pH 8.0) was incubated at 37°C for variable periods, dialyzed at 4°C and lyophilized. To determine the molar ratio of lactose/BSA, lactose content was measured by the phenol/sulfuric acid method (Dubois et al., 1956) and BSA was assayed by a slight modification of the Lowry method (Lowry et al., 1951). In this study, two types of galactosylated BSA were further used for conjugation with MTX; one with the lactose/BSA molar ratio of 5:1 (L_5BSA), the other with the ratio of 24:1 (L_{24}BSA). When stored at −10°C, galactosylated BSA was stable for at least 1 year without any changes in sugar content.

2.2.2. Conjugation of MTX to galactosylated BSA

MTX was conjugated to BSA or galactosylated BSA (L_5BSA, L_{24}BSA) by a slight modification of the method of Kim and Oh (1988). A mixture of 12.6 mM MTX and 0.25 mM (galactosylated) BSA was acidified to pH 6.0 with 0.1 N HCl, and slowly added with EDC (final concentration, 65 mM). In some experiments, [^{3}H]MTX (96.2 μCi) was added to the mixture of MTX. The reactants were stirred overnight at 4°C and the product was separated from unreacted molecules by ultrafiltration and then lyophilized. The amount of MTX per conjugate was determined by measuring its absorbance at 373 nm for MTX and 660 nm for BSA by the Lowry method. For [^{3}H]MTX-containing conjugates, the content of MTX was measured by counting the radioactivity of [^{3}H]MTX in a liquid scintillation counter (Rack Beta, LKB-Wallac, Turku, Finland). The molar ratio of MTX to the carrier protein was in the range of 10–12 regardless of the degree of galactosylation of BSA.

2.3. In vitro uptake of MTX conjugates by hepatocytes

2.3.1. Preparation of hepatocytes

Male Wistar Albino rats (180–200 g, Experimental Animal Breeding Center of Seoul National University, Seoul, South Korea) were used as the source of hepatocytes, which were harvested according to the method of Seglen (1976). The liver was first perfused through the portal vein with Hanks balanced salt solution (HBSS) and removed to a recirculating liver perfusion system (Dunn et al., 1979). After HBSS was recirculated for 5 min, 0.05% collagenase in HBSS with 4 mM $CaCl_2$ was perfused for 15 min. The liver was then minced, diluted with 20 vol. of HBSS and filtered successively through gauze. Hepatocytes were purified by three cycles of sedimentation and resuspended in HBSS to 1×10^5 cells/ml. The tryphan blue test showed that at least 85% of the cells were viable.

2.3.2. Hepatocytes uptake

The mixtures of [^{3}H]MTX and MTX in free or conjugated forms were incubated with 10 ml of hepatocytes (1×10^5 cells/ml) at 37°C. During this incubation period, 0.5 ml of the suspension was collected at certain time points, mixed with 20 μl of 2 N HCl to stop the uptake and centrifuged at 3000 rpm for 5 min. The pellet of hepatocytes was resuspended and washed twice with pH 7.4 phosphate buffer(PB) by centrifugation. The cell pellet was then dissolved in Soluene-350®. The amount of cell-associated MTX was determined by measuring the radioactivity of the cell lysates.

2.4. In vitro release of MTX from the conjugates

Conjugate forms of MTX equivalent to 2 mg of free MTX were incubated at 37°C in 50 ml of PB (pH 7.4), 20 ml of rat plasma, or 20 ml of rat liver homogenates. At each time point, 0.1-ml aliquots were sampled in duplicate, deproteinized with acetonitrile and subjected to HPLC assay for MTX at 313 nm (Chen and Chiou, 1981).

2.5. In vivo pharmacokinetics and tissue distribution

The mixtures of [^{3}H]MTX and MTX in free or conjugated forms (equivalent to 5 mg/ml MTX) were injected into male Wistar Albino rats via the femoral vein. At each time point, blood samples were collected from the femoral artery and in parallel, rats were sacrificed to obtain various organs. Blood samples were centrifuged immediately to avoid the potential 'blood storage effect' that can interfere with the MTX assay in plasma. Tissue samples were rinsed with cold saline, blotted dry and weighed. Total radioactivities in three forms of [^{3}H]MTX (free, conjugated and metabolized forms) contained in each tissue sample were measured by a scintillation counter. Amounts of intact form of MTX were determined by HPLC.

2.5.1. Pharmacokinetic analysis

Area under the plasma concentration–time curve from time zero to 24 h ($AUC_{0\rightarrow 24h}$) was calculated by the trapezoidal method. Mean residence time (MRT), time-averaged total body clearance (CL) and apparent volume of distribution at steady state (V_{ss}) were calculated using the noncompartmental analysis method (Gibaldi and Perrier, 1982).

2.5.2. Evaluation of liver targetability

The targeted delivery of MTX to the liver was evaluated using two indices based on the area under the total amount of radioactivity–time curve (AUQ); one is the weight-averaged overall drug targeting efficiency (Te^*, Gallo et al., 1989); the other is the weight-averaged drug targeting efficiency against a given non-targeting tissue (te^*, Gupta and Hung, 1989; Kim and Jeong, 1997).

$$Te^* = \frac{AUQ_i}{\sum(AUQ)_j} \times 100$$

$$te^* = \frac{AUQ_i}{AUQ_{\text{non-target tissue}}}$$

where i is the target organ and j is each organ.

2.6. Statistical analysis

ANOVA was used to analyze data for statistical significance ($P < 0.05$).

3. Results and discussion

3.1. In vitro uptake of MTX conjugates by hepatocytes

To test whether the hepatocyte delivery of MTX can be enhanced by galactose receptor-mediated endocytosis, the uptake of variously galactosylated conjugates was studied at the cellular level. In this study, hepatocytes were chosen as target cells of MTX–galactosylated BSA conjugates since these are known to be the principal cells of the liver involved in the clearance of proteins with galactosyl ligands (Wall et al., 1980). The least amount of cellular uptake was observed in free MTX (Fig. 1). The uptake increased as MTX-carrying BSA conjugated with a greater number of galactose, implying that the increased uptake of the conjugates was via galactose receptors on hepatocytes. The extent of galactosylation also affected the cellular uptake rates of the conjugates. Free MTX, MTX–BSA and MTX–L_5BSA showed significant uptake only during the initial period of incubation, whereas MTX–L_{24}BSA conjugates showed a gradual increase of uptake throughout the test period. The enhanced uptake of MTX–L_{24}BSA conjugates appears to be due to the higher affinity of L_{24}BSA to the galactose receptors on the hepatocytes (Krantz et al., 1976).

3.2. In vitro release of MTX from conjugates

Conjugated MTX may have a lower binding affinity to dihydrofolate reductase, the target enzyme of MTX (Fitzpatrick and Garnett, 1995). To minimize the side effects of MTX on other sites in the body, MTX conjugates thus need to be stable in the blood as an inactive conjugate form. However, once the conjugates have reached the liver, they should liberate MTX as a free, therapeutically active form. In this regard, we evalu-

ated the release of MTX from its variously galactosylated conjugates in different environments: PB, rat plasma and liver homogenate. PB was used as a control medium. Rat plasma and liver homogenate were used as the model system to test the stability of the conjugates in the blood and the liberation of MTX from its conjugates in the liver, respectively.

The release rates of MTX from its conjugates were not affected by the extent of galactosylation of BSA (Fig. 2). In all three conjugates, MTX–BSA, MTX–L_5BSA and MTX–L_{24}BSA, liver homogenates (Fig. 2C) significantly accelerated the release of MTX as compared with PB (Fig. 2A) and rat plasma (Fig. 2B). In liver homogenates, MTX was liberated in a biphasic pattern of an initial rapid release over 2 h followed by slower release. At 24 h of incubation in liver homogenate under current experimental conditions, the percentage release of MTX from BSA, L_5BSA, and L_{24}BSA conjugates was approximately 11.5 ± 0.75, 12 ± 0.53, and 12.7 ± 0.80, respectively.

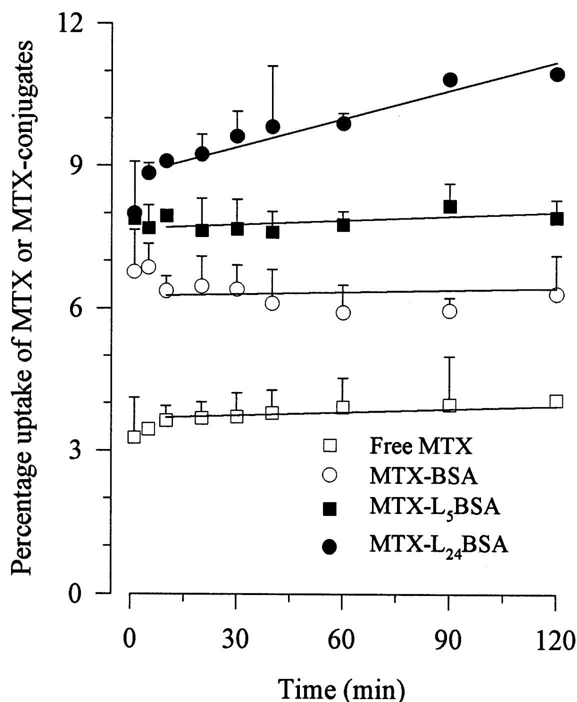

Fig. 1. Uptake of free MTX and MTX-conjugates by rat hepatocytes. The mixtures of [^{3}H]MTX and MTX in free or conjugate forms were incubated with rat hepatocytes at 37°C. At indicated time points, the amount of cell-associated MTX or MTX-conjugate was determined by the radioactivity of cell lysates. Each point represents the mean value ± S.D. ($n = 5$).

We observed that these conjugates having similar contents of MTX (10–12 MTX per conjugate) showed similar release rates of MTX. This observation is in good agreement with the previous report that the release rates of MTX from its conjugates is controlled by the quantity of MTX in the conjugates (Halbert et al., 1987; Kim et al., 1989). The slower release of MTX in rat plasma reflects the stability of MTX conjugates in the blood, which is the desired property for lowering the side effects. Furthermore, the higher stability of conjugates in the blood could greatly enhance the delivery of conjugates to the target place.

The biphasic release pattern in liver homogenates could be interpreted such that ‘physically adsorbed’ and ‘covalently bound’ MTX contributed to the initial rapid release and the slow later phase, respectively (Kim et al., 1989; Yoon et al., 1991). It is thought that lysosomal enzymes abundant in liver homogenates might have played a role in releasing covalently bound MTX from the conjugates (Ciechanover et al., 1983). The slow gradual release of covalently bound MTX in liver homogenates suggests the potential of conjugates as sustained delivery systems in the target organ.

3.3. Pharmacokinetics

Based on the in vitro hepatocyte uptake of MTX (Fig. 1), we chose MTX–L_{24}BSA as the most promising conjugate for in vivo study and compared the pharmacokinetics of MTX-equivalent as [^{3}H]MTX delivered in L_{24}BSA conjugates with those delivered as the free form of [^{3}H]MTX or [^{3}H]MTX–BSA conjugates. MTX-equivalent was determined based on the total radioactivity of [^{3}H]MTX, which cannot differentiate between the conjugated/liberated [^{3}H]MTX or the unmetabolized/metabolized [^{3}H]MTX. [^{3}H]MTX–L_{24}BSA conjugates showed clearly different pharmacokinetics from free [^{3}H]MTX and [^{3}H]MTX–BSA conjugates (Fig. 3). The plasma level of free [^{3}H]MTX most rapidly declined in a biexponential fashion with an apparent terminal half-life of 0.35

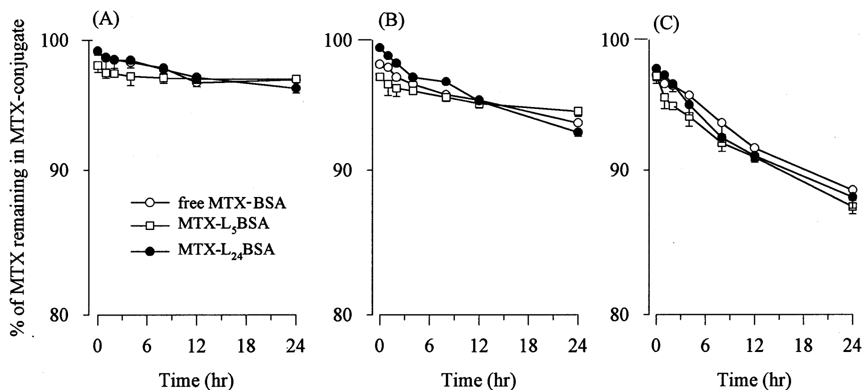

Fig. 2. In vitro release of MTX from three different conjugates of MTX. MTX–BSA, MTX–L_5BSA and MTX–L_{24}BSA were incubated at 37°C in 50 ml of PB (A), 20 ml of rat plasma (B), or 20 ml of rat liver homogenates (C). The amount of MTX released at each time was assayed by HPLC at 313 nm. Each point represents the mean value ± S.D. ($n = 5$).

h. [^{3}H]MTX administered in BSA conjugates showed the slowest decline with an apparent terminal half-life of 6 h. In contrast, [^{3}H]MTX–L_{24}BSA showed a biphasic pattern; a rapid distributive phase with a half-life of 0.57 h, and a subsequent slow terminal phase. The pharmacokinetic parameters of these three groups are summarized in Table 1.

The lowest *CL* of [^{3}H]MTX–BSA seems to be a result of two factors; first, relatively lower uptake of [^{3}H]MTX–BSA by hepatocytes (Fig. 1), second, the high molecular weight of [^{3}H]MTX–BSA conjugates since the molecular size has been shown to be a limiting factor for renal excretion (Data and Nies, 1974). [^{3}H]MTX–L_{24}BSA showed about fivefold higher *CL* than [^{3}H]MTX–BSA. Considering the similar molecular size of [^{3}H]MTX–L_{24}BSA to [^{3}H]MTX–BSA and the low distribution of [^{3}H]MTX–L_{24}BSA to the kidney (Table 2), it is unlikely that [^{3}H]MTX–L_{24}BSA would have a higher renal clearance than [^{3}H]MTX–BSA. Rather, it appears to be more feasible that the higher *CL* of [^{3}H]MTX–L_{24}BSA might be due to the preferential uptake by the liver via galactose receptors on hepatocytes.

3.4. Tissue distribution and liver targetability

Free [^{3}H]MTX, [^{3}H]MTX–BSA and [^{3}H]MTX–L_{24}BSA showed significantly different tissue distribution patterns (Table 2). In the spleen, heart, kidney and lung, Te^* values decreased in the order of free [^{3}H]MTX, [^{3}H]MTX–BSA and [^{3}H]MTX–L_{24}BSA. However, in the plasma, [^{3}H]MTX–BSA showed the highest Te^* value; five- and sixfold higher than that of free [^{3}H]MTX and [^{3}H]MTX–L_{24}BSA, respectively. Such a high Te^* of [^{3}H]MTX–BSA in the plasma seems to have resulted from the lowest *CL* as shown in Table 1.

The liver targetability of MTX in various forms was evaluated with two indices, Te^* and te^*, based on the tissue distribution of [^{3}H]MTX (Table 2). Free [^{3}H]MTX and [^{3}H]MTX–BSA showed similar Te^* values in the liver (Te^*_{liver}). In contrast, [^{3}H]MTX–L_{24}BSA showed 87.5% of $\mathrm{Te}^*_{\text{liver}}$, almost twofold higher than others, which appears to be contributed by the superior uptake of [^{3}H]MTX–L_{24}BSA by the hepatocytes (Fig. 1). This result indicates that the liver targetability of MTX could be substantially enhanced by administering MTX in the form of conjugate, MTX–L_{24}BSA.

As another index of testing the liver targetability against undesirable targeting organ, te^* was used. By defining the denominator of te^* formula as an organ that undergoes the most severe toxicity, we may predict the balance between the therapeutic effect and the toxicity of various MTX forms. In this study, we defined te^* as the ratio of AUQ_{liver} to AUQ_{kidney} since MTX is known to have severe toxicity mainly in the kidney (Shen

and Azarnoff, 1978). Though free [^{3}H]MTX and [^{3}H]MTX–BSA did not significantly differ in Te^*_{liver}, they showed a significant difference in te^*. The te^* of [^{3}H]MTX–BSA was about sixfold higher than that of free [^{3}H]MTX, which seems to be due to diminished distribution of higher molecular weight [^{3}H]MTX–BSA to the kidney. [^{3}H]MTX–L_{24}BSA showed a 25-fold increase in te^* compared with free [^{3}H]MTX. The highest te^* value of MTX–L_{24}BSA suggests that MTX given in the L_{24}BSA conjugate would exert maximal therapeutic effects on the target organ, liver, but minimal toxic effects on the kidney.

3.5. Tissue levels of intact MTX

Above, both Te^* and te^* were measured based on the total radioactivity of [^{3}H]MTX, which cannot differentiate between the conjugate/liberated [^{3}H]MTX or the unmetabolized/metabolized [^{3}H]MTX. Since it is known that bound or metabolized MTX loses its affinity to the target enzyme, dihydrofolate reductase (Shen and Azarnoff, 1978), the amount of unbound and intact forms of MTX will be a more accurate indicator of therapeutic/toxic effects. Thus, to have more insight on the therapeutic/toxic effects of the various forms of MTX, the tissue concentration–time profiles of MTX in its intact form were measured by HPLC.

In the liver, free MTX showed the highest level of intact MTX at 5 min after injection followed by the fastest decline. Such a fast disappearance of intact MTX might have resulted from the highest accessibility of free MTX to its metabolizing enzymes. MTX given in MTX–BSA conjugates showed the lowest level of intact MTX for 120 min after injection. MTX administered in MTX–L_{24}BSA showed a gradual increase of intact MTX over 4 h (Fig. 4A), suggesting that MTX–L_{24}BSA may exhibit therapeutic activity in the liver for a prolonged period as a sustained delivery system of MTX.

On the other hand, in the kidney, free MTX showed the highest level of intact MTX for 120 min after injection (Fig. 4B). MTX–BSA and

Table 1
The noncompartmental pharmacokinetic parameters of total radioactivity as expressed in terms of MTX-equivalent after i.v. injection of free [^{3}H]MTX, [^{3}H]MTX–BSA and [^{3}H]MTX–L_{24}BSA conjugates to rats[a]

Parameter	Free [^{3}H]MTX	[^{3}H]MTX–BSA	[^{3}H]MTX–L_{24}BSA
AUC (μg h ml^{-1})	16.6 ± 3.03	898 ± 44.7**	189 ± 33.8**
MRT (h)	0.410 ± 0.091	8.42 ± 0.606*	0.790 ± 0.103*
V_{ss} (ml kg^{-1})	245 ± 20.6	93.7 ± 3.36**	42.3 ± 3.46**
CL (ml h^{-1} kg^{-1})	626 ± 130	11.2 ± 0.570**	54.7 ± 9.77**

[a] Values are mean ± S.D. ($n = 4$).
* $P < 0.01$ when compared with the values for free MTX.
** $P < 0.001$ when compared with the values for free MTX.

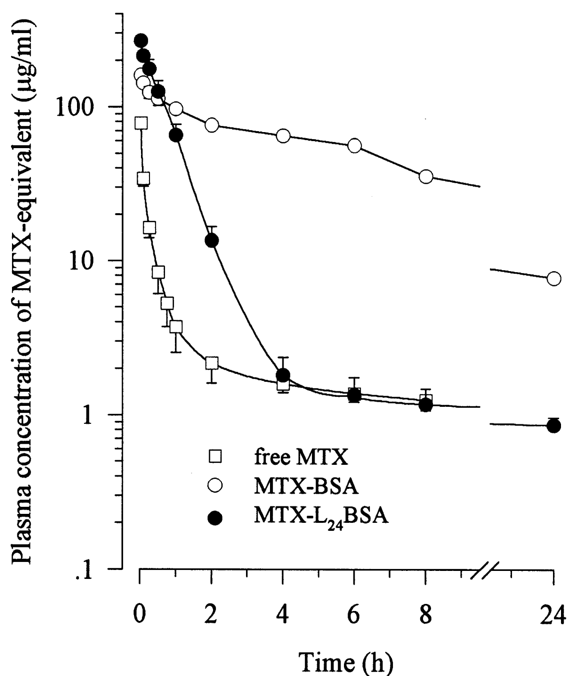

Fig. 3. Plasma concentration–time profiles of MTX-equivalent following intravenous administration of the mixtures of [^{3}H]MTX and MTX in free or conjugate forms. Blood samples were collected at indicated time points. Total amounts of MTX were measured by ^{3}H radioactivity, and divided by initial dose to obtain the percentage dose. Each point represents the mean percentage dose ± S.D. ($n = 4$).

Table 2
The AUQ_{24h} (μg day) and Te^* of total radioactivity as expressed in terms of MTX-equivalent after i.v. injection of free [^{3}H]MTX, [^{3}H]MTX–BSA and [^{3}H]MTX–L_{24}BSA conjugates to rats

	Free [^{3}H]MTX		[^{3}H]MTX–BSA		[^{3}H]MTX–L_{24}BSA)	
	AUQ_{24h} (μg day)	Te^* (%)	AUQ_{24h} (μg day)	Te^* (%)	AUQ_{24h} (μg day)	Te^* (%)
Liver	1240	49.2	10 600	42.2	31400	87.5
Spleen	223	8.85	337	1.34	463	1.29
Heart	39.2	1.56	188	0.746	27.0	0.0752
Kidney	683	27.1	889	3.53	682	1.90
Lung	85.6	3.40	600	2.38	349	0.972
Plasma[a]	252	10.0	12 600	50.0	2980	8.30
Total	2520	100	25 200	100	35 900	100
Te[b]	1.81		11.9		46.0	

[a] Total blood volumes were considered as 6 ml per 100 g body wt of rat.
[b] Ratio of AUQ_{liver} to AUQ_{kidney}.

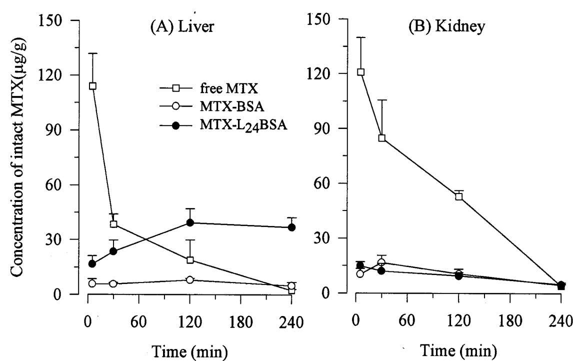

Fig. 4. Tissue concentration–time profiles of intact MTX in the liver (A) and kidney (B). MTX in various forms were intravenously injected into rats. At indicated time points, rats were sacrificed and the concentrations of intact MTX in the liver and kidney were determined by HPLC. Each point represents the mean value ± S.D. ($n = 4$).

MTX–L_{24}BSA did not show significant differences in the kidney levels of intact MTX. Much lower concentrations of intact MTX observed in these conjugates indicate that MTX–L_{24}BSA can also minimize the toxicity of MTX in the kidney.

In conclusion, our results provided evidence that MTX–galactosylated albumin conjugates could enhance hepatocyte uptake and liver targetability of MTX with minimal kidney distribution. Moreover, the gradual increase of intact MTX in the liver indicates the possibility of developing MTX–galactosylated albumin conjugates as sustained delivery systems. These results suggest that galactosylated albumin could be further developed as promising liver-targeting carriers of other drugs for liver diseases.

Acknowledgements

This study was supported in part by a research grant from (HMP-96-D-2-0024) of the ’96–97 Good Health R&D Project, Ministry of Health & Welfare, South Korea.

References

Anderson, J.H., Warren, H.W., McArdle, C.S., 1994. Clinical pharmacokinetic advantages of new drug delivery methods for the treatment of liver tumors. Clin. Pharmacokinet. 27, 191–201.
Ashwell, G., Harford, J., 1982. Carbohydrate-specific receptors of the liver. Annu. Rev. Biochem. 51, 531–554.
Chen, M.L., Chiou, W.L., 1981. Sensitive and rapid high-performance liquid chromatographic method for the simultaneous determination of methotrexate and its metabolites in plasma, saliva and urine. J. Chromatogr. 226, 125–134.
Ciechanover, A., Schwartz, A.L., Lodish, H.F., 1983. Sorting and recycling cell surface receptors and endocytosed ligands: the asialoglycoprotein and transferrin receptors. J. Cell. Biochem. 23, 107–130.
Data, J.L., Nies, A.S., 1974. Dextran 40. Ann. Intern. Med. 81, 500–504.

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

Dunn, W., Labadie, J., Aronson, N., 1979. Inhibition of ^{125}I-asialofetuin catabolism by leupeptin in the perfused rat liver and in vivo. J. Biol. Chem. 254, 4191–4196.

Fallon, R.J., Schwartz, A.L., 1988. Asialoglycoprotein receptor phosphorylation and receptor-mediated endocytosis in hepatoma cells. Effect of phorbol esters. J. Biol. Chem. 263, 13159–13166.

Fiume, L., Mattioli, A., Busi, C., Spinosa, G., Wieland, T., 1982. Conjugates of adenine 9-alpha-D-arabinofuranoside monophosphate (ara-AMP) with lactosaminated homologous albumin are not immunogenic in the mouse. Experientia 38, 1087–1089.

Fitzpatrick, J.J., Garnett, M.C., 1995. Design, synthesis and in vitro testing of methotrexate carrier conjugates linked via oligopeptide spacers. Anticancer Drug Des. 10, 1–9.

Gallo, J.M., Gupta, P.K., Hung, C.T., Perrier, D.G., 1989. Evaluation of drug delivery following the administration of magnetic albumin microspheres containing adriamycin to the rat. J. Pharm. Sci. 78, 190–194.

Gibaldi, M., Perrier, D., 1982. Pharmacokinetics, 2nd edn. Marcel Dekker, New York.

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

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

Johnson, P.J., 1993. Hepatitis viruses, cirrhosis, and liver cancer. J. Surg. Oncol. Suppl. 3, 28–33.

Kim, C.-K., Han, J.-H., 1995. Lymphatic delivery and pharmacokinetics of methotrexate after intramuscular injection of differently charged liposome-entrapped methotrexate to rats. J. Microencapsul. 12, 437–446.

Kim, C.-K., Hwang, S-J., 1993. Pharmacokinetics and organ-distribution of ^{3}H-methotrexate and ^{3}H-methotrexate-human serum albumin conjugates in mice. Drug Dev. Ind. Pharm. 19, 961–970.

Kim, C.-K., Jeong, E.J., 1997. Enhanced lymph node delivery and immunogenicity of hepatitis B surface antigen entrapped in galactosylated liposomes. Int. J. Pharm. 147, 143–151.

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

Kim, C.-K., Lee, M.G., Park, M.K., Lee, H.J., Kang, H.J., 1989. In vitro release characteristics of methotrexate-bovine serum albumin and 5-fluorouracil acetic acid-human serum albumin conjugates. Arch. Pharm. Res. 12, 186–190.

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

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

Krantz, M.J., Holtzman, N.A., Stowell, C.P., Lee, Y.C., 1976. Attachment of thioglycosides to proteins: enhancement of liver membrane binding. Biochemistry 15, 3963–3968.

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

Rettinger, S.D., Kennedy, S.C., Wu, X., Saylors, R.L., Hafenrichter, D.G., Flye, M.W., Ponder, K.P., 1994. Liver-directed gene therapy: quantitative evaluation of promotor elements by using in vivo retroviral transduction. Proc. Natl. Acad. Sci. USA 91, 1460–1464.

Schwartz, A.L., Rup, D., Lodish, H.F., 1980. Difficulties in the quantification of asialyglycoprotein receptors on the rat hepatocyte. J. Biol. Chem. 255, 9033–9036.

Schwartz, B.A., Gray, G.R., 1977. Proteins containing reductively aminated disaccharides. Synthesis and chemical characterization. Arch. Biochem. Biophys. 181, 542–549.

Seglen, P.O., 1976. Preparation of isolated rat liver class. Methods Cell Biol. 13, 29–83.

Sett, R., Sarkar, H.S., Das, P.K., 1993. Pharmacokinetics and biodistribution of methotrexate conjugated to manosyl human serum albumin. J. Antimicrob. Chemother. 31, 151–159.

Seymour, L.W., Ulbrich, K., Wedge, S.R., Hume, I.C., Strohalm, J., Duncan, R., 1991. *N*-(2-Hydroxypropyl)methacrylamide copolymer targeted to the hepatocyte galactose-receptor: pharmacokinetics in DBA2 mice. Br. J. Cancer 63, 859–866.

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

Tucker, L.E., 1992. Primary biliary cirrhosis: current diagnosis and treatment. Gastroenterol. Nursing 15, 70–77.

Wall, D.A., Wilson, G., Hubbard, A.L., 1980. The galactose-specific recognition system of mammalian liver; the route of ligand internalization in rat hepatocytes. Cell 21, 79–83.

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