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Pharmacokinetics of methotrexate after intravenous infusion of methotrexate-rabbit serum albumin conjugate to rabbits

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Summary

The pharmacokinetics of methotrexate (MTX) were compared after 30 min intravenous infusion of the same dose (10 mg/kg as MTX) of MTX (treatment I) or MTX-rabbit serum albumin (RSA) conjugate (treatment II) to rabbits. In treatment II, the mean peak plasma level of MTX was significantly lower (48.1 vs 13.8 μg/ml), and plasma levels declined more slowly thereafter (mean apparent half-lives of 3.26 vs 4.96 h) than those in treatment I. In treatment II, the values of AUC (2360 vs 1510 μg min ml^{-1}) and CL_R (2.49 vs 0.452 ml min^{-1} kg^{-1}) were decreased, however, the values of V_{SS} (0.311 vs 1.47 l/kg), MRT (1.62 vs 3.71 h), $t_{1/2}$ (3.26 vs 4.96 h), and CL_{NR} (1.66 vs 6.13 ml min^{-1} kg^{-1}) were significantly increased. The above data suggested that MTX resides longer in the rabbit, and that nonrenal metabolism of MTX increases in treatment II. It could be explained by the fact that MTX is released slowly from MTX-RSA conjugate, and that the disposition of MTX is saturable. The amounts of MTX (μg/g tissue) remaining in kidney, stomach, small intestine, and large intestine after 30 min infusion of MTX-RSA conjugate were 33, 6.1, 3.1, and 10 times lower, respectively, than those after 30 min infusion of free MTX. It might suggest that the administration of MTX-SA conjugate has less side effects of MTX in these organs or tissues than those of free MTX. The in vitro release of MTX from MTX-RSA conjugate in phosphate buffer of pH 7.4, the buffer with protease, rat liver homogenate, or human plasma was biphasic process. For example, an initial rapid release over approx. 6 h appears to be due to physically adsorbed MTX with the slower secondary release due to covalently bound drug. The release of MTX from the conjugate in vitro was accelerated in the presence of protease or liver homogenate.

Introduction

Methotrexate (MTX), a folic acid antagonist, has been widely used in chemotherapy of various types of neoplastic diseases (Chabner et al., 1975). Recently, high dose MTX (HDMTX) therapy with leucovorin rescue factor has been commonly employed. In HDMTX therapy, MTX, 1–30 g per m^2 body surface area is usually infused over a long period of time (e.g., 6 h) to maintain adequate concentrations of MTX in cancer cells; this, however, has led to an increase in the incidence of systemic side effects or toxicities to the GI tract, kidney, and bone marrow (Evans et al., 1986).

The ideal dosage form in cancer chemotherapy

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is that which provides a specific delivery of anticancer agent to the tumor site in sufficient amounts for a long period of time with minimum systemic side effects to the GI tract, kidney, and bone marrow (Yoshioka et al., 1981). For this purpose, anticancer drug-macromolecule (such as serum albumin or polypeptide) conjugates have been synthesized (Chu and Whiteley, 1977; Ryser and Shen, 1978; Halbert et al., 1987) and their in vitro anticancer activities were reported (Chu and Whiteley, 1977, 1979; Chu and Howell, 1981a,b; Kato et al., 1982; Trouet and Masquelier, 1982; Roos et al., 1984; Halbert et al., 1987). For example, the MTX-bovine serum albumin (BSA) conjugate increased the survival time of mice more than did free MTX when injected intraperitoneally into mice bearing the ascitic form of L1210 (Jacobs et al., 1971), and the conjugate was proved more effective than free MTX against the development of lung metastasis from a subcutaneously transplanted Lewis lung carcinoma (Chu and Whiteley, 1979). The results obtained with the albumin conjugates coupled with the fact that serum albumin has been demonstrated to accumulate at tumor sites (Cerrottini and Isliker, 1967; Chu and Whiteley, 1977) led us to study MTX-rabbit serum albumin (RSA) conjugate as a means of reducing the systemic toxicities and increasing the therapeutic benefit (as drug targeting agents) of MTX. The pharmacokinetics of anticancer drug-macromolecular conjugates have received only scant attention in the literature since very few studies have been reported.

The purpose of this paper is to report our results on the pharmacokinetics and tissue distribution of MTX after 30 min of intravenous infusion of the equivalent dose (10 mg/kg as MTX) of free MTX and MTX-RSA conjugate to rabbits. Release of MTX from MTX-RSA conjugates in phosphate buffer of pH 7.4, buffer with protease, rat liver homogenates, and human plasma was also investigated.

Materials and Methods

Synthesis of MTX-RSA conjugate

MTX-RSA conjugate was synthesized by the carbodiimide reaction, and the contents of MTX in the conjugate were measured by a method similar to that reported for MTX-human serum albumin (HSA) conjugate (Kim and Oh, 1988). The conjugate contained approx. 3% w/w of MTX, and similar result was also obtained from 'low-strength' MTX-BSA conjugates (Halbert et al., 1987). Recently, it was reported (Halbert and Florence, 1989) that MTX-BSA conjugates are heterogeneous compounds containing mixtures of albumin and albumin polymers with varying quantities of covalently bound drug.

Release of MTX from MTX-RSA conjugate

A weight of MTX-RSA conjugate equivalent to 2 mg of MTX was dissolved in 50 ml of phosphate buffer of pH 7.4, the buffer containing 200 mg of protease (activity 31 400 U/g at pH 8.0, kindly supplied by Dong-A Pharm. Co., Seoul, Korea), rat liver homogenate, and human plasma, respectively. The rat liver was homogenized by adding 5 volumes of phosphate buffer (Graham, 1984; Kim et al., 1989). The mixture was incubated in a water-bath shaker at 37 °C and 50 oscillations per min (opm). Two 0.1-ml samples were collected at appropriate time intervals and stored in a freezer prior to HPLC analysis of MTX.

Animals

Sixteen male, New Zealand White rabbits (A–P, 1.50–2.10 kg, Korea Laboratory Animal Development, Seoul, Korea) were anesthetized with 50–100 mg of intravenous ketamine (kindly supplied by Yu-Han Pharm. Co., Seoul, Korea). The carotid artery and jugular vein were catheterized with silastic tube (Dow Corning, Midland, MI). The cannulas were exteriorized on the dorsal side of the neck where each cannula terminated in a three-way stopcock. The animals were allowed to recover for 4–5 h before study. Urine samples were collected using pediatric Foley Catheter (Dover, Searl Medical Products, U.S.A., Inc., Dallas, TX) which was introduced into the urinary bladder.

Intravenous infusion study

MTX (50 mg vial, kindly supplied by Yu-Han Pharm. Co.; dissolved in 25 ml of 0.9% NaCl sterile solution), 10 mg/kg, and MTX-RSA con-

jugate (freshly reconstituted with 25 ml of the NaCl solution), 10 mg/kg as MTX, were infused over 30 min using an infusion pump (Microfeeder, Furue Science Co., Japan) through the jugular vein of rabbits A–D (treatment I) and E–H (treatment II), respectively. The blood samples were withdrawn (1 ml each) via the carotid artery, and 2 ml of heparinized normal saline solution (10 U/ml) was used for flushing the cannula after each blood sampling. The blood sampling times were −30 (to serve as a control), −20, −10, 0 (the end of infusion), 10, 20, 30, 45, 60, 90, 120, 180, 240, 360, 480, and 720 min after the dose. The blood samples were centrifuged immediately to avoid or minimize the potential 'blood storage effect' in the determination of plasma MTX concentration (Lee et al., 1984, 1986). Two 0.1-ml aliquots of plasma sample were stored in the freezer prior to HPLC analysis of MTX. Urine samples were collected at −30–0, 0–30, 30–60, 60–90, 90–120, 120–180, 180–240, 240–360, 360–480, 480–720, and 720–1440 min after the dose. At the end of each interval, the bladder was flushed twice with 20 ml of distilled water, and 20–40 ml of air to ensure complete recovery of urine. The washings were then combined with the urine, and the total volumes were measured. Two 0.1-ml aliquots of the combined urine were stored in the freezer prior to HPLC analysis of MTX.

Tissue distribution study

The same doses (containing 10 mg/kg as MTX) of MTX and MTX-RSA conjugate were similarly infused to rabbits I–M (treatment III) and rabbits N–P (treatment IV), respectively. After 30 min of infusion, whole blood was collected as much as possible through the carotid artery, and the rabbit was exsanguinated. Brain, heart, lung, liver, spleen, stomach, and kidney were obtained and weighed. Approx. 1 g of liver, kidney, heart, lung, small intestine, large intestine, stomach, muscle, fat, brain, spleen or mesentery was quickly removed, rinsed, minced and homogenized with 4 volumes of 0.2 M NaOH (Chen and Chiou, 1982) in a tissue homogenizer (Tissuemizer, Tekman Co., Model SDT-1810 Cincinnati, OH), and centrifuged immediately. Bile juice and plasma were also diluted with 4 volumes of 0.02 M NaOH. Two 0.1-ml aliquots of the diluted bile juice or plasma, and the supernatant of tissue homogenates were stored in the freezer prior to HPLC analysis of MTX.

HPLC analysis

The concentrations of MTX in this study were measured by a slight modification (Lee et al., 1984) of a reported HPLC method (Chen and Chiou, 1981). An aliquot of supernatant was injected onto the column after deproteinization of a 0.1 ml sample with 2.5 volumes of acetonitrile. Peak height measurements were used for quantitation. In this study, concentrations of MTX-RSA conjugate were not measured.

Pharmacokinetic analysis

The area under the plasma concentration-time curves from time zero to time infinity (AUC) was calculated by the trapezoidal rule-extrapolation method (Chen et al., 1982); this method employs the logarithmic trapezoidal rule, recommended by Chiou (1978) for the calculation of area during the declining plasma-level phase, and the linear trapezoidal rule for the rising plasma-level phase. The area from the last data point to infinity was estimated by dividing the last concentration by the apparent terminal rate constant.

Standard methods (Riegelman and Collier, 1980; Chen et al., 1982) were used to calculate the following parameters; time-averaged total body clearance (CL), area under the first moment of plasma concentration-time curve (AUMC), mean residence time (MRT), volume of distribution at steady state (V_{SS}), time-averaged renal clearance (CL_R), and nonrenal clearance (CL_{NR}):

$$CL = \text{dose}/AUC \tag{1}$$

$$AUMC = \int_0^\infty t\, C_P\, dt \tag{2}$$

$$MRT = AUMC/AUC - T/2 \tag{3}$$

$$V_{SS} = CL \times MRT \tag{4}$$

$$CL_R = X_U/AUC \tag{5}$$

$$CL_{NR} = CL - CL_R \tag{6}$$

where C_P is the plasma concentration at time t, and T is the infusion time. X_U is the amount of MTX excreted in the urine up to time infinity (this was assumed to equal the total amounts excreted over 24 h, since negligible amounts of MTX could be found in urine collected later). The mean values of each clearance, V_{SS}, and half-life were calculated by the harmonic mean method (Chiou, 1979).

Statistical analysis

The data were analyzed for statistical significance ($p < 0.05$) by unpaired t-test.

Results and Discussion

Fig. 1 shows the percentages of MTX remaining in MTX-RSA conjugate after incubation with the various solutions. In all the solutions studied,

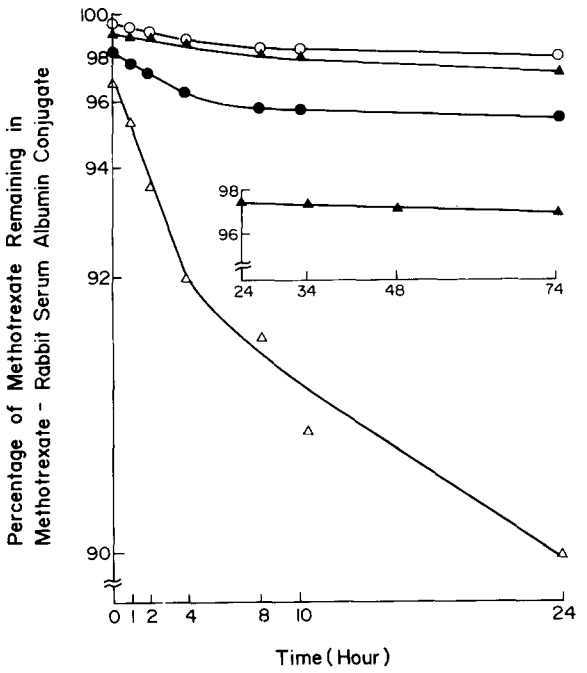

Fig. 1. Percentages of methotrexate remaining in methotrexate-rabbit serum albumin conjugate after incubation of the conjugate in water-bath shaker at 37 °C and 50 oscillations per min with phosphate buffer of pH 7.4 (▲), buffer with protease (●), rat liver homogenate (△) and human plasma (○), respectively. The inset shows the percentages incubated with phosphate buffer of pH 7.4 from 24 h to 74 h.

TABLE 1

Release rate constants of MTX from MTX-RSA conjugate

	Rate constant ($k \times 10^{-5}$) (h^{-1})	
	Initial	Second
Plasma	156	40.4
Phosphate buffer, pH 7.4	267	74.0
Phosphate buffer, pH 7.4 in the presence of protease	757	8.65
Liver homogenate	1930	96.5

the MTX-RSA conjugate displayed an initial fast release period up to approx. 6 h, and thereafter the release rate was very much slower. The rate constants determined from the release experiments are listed in Table 1. The initial rate constants are 3.9, 3.6, 88, and 20 times greater than the second rate constants in human plasma, phosphate buffer of pH 7.4, buffer with protease, and rat liver homogenate, respectively. It should be noted that protease and liver homogenate accelerated the release of MTX from the conjugate.

The data presented in Fig. 1 demonstrate that release of MTX from the conjugate is a biphasic process, which suggested that the MTX attaches to the RSA by two distinct types of linkage. An initial rapid release over 6 h appears to be due to 'physically adsorbed' MTX with the slower secondary release due to 'covalently' bound drug as shown from MTX-BSA conjugate (Halbert et al., 1987) and MTX-HSA conjugate (Kim et al., 1989). MTX is known to bind physically to albumin to the extent of about 46% at normal clinical drug levels (Paxton, 1981).

The mean MTX plasma concentration-time curves from treatments I and II are shown in Fig. 2. After infusion of free MTX (treatment I), the plasma concentrations of MTX rose rapidly during infusion with a mean peak concentration (at time 0) of 48.1 μg/ml and decreased poly-exponentially post-infusion with a mean apparent terminal half-life of 3.26 h. However, the plasma concentration profiles were quite different after infusion of MTX-RSA conjugate (treatment II); the plasma concentrations rose slowly during infusion with a mean peak concentration of 13.8 μg/ml

and declined poly-exponentially post-infusion with a mean apparent terminal half-life of 4.96 h. Similar half-lives, 2.83 and 5.66 h for treatment I and II, respectively, were also obtained from 24 h urinary excretion rate data (Fig. 3). The slowly increasing MTX concentrations during infusion and slowly declining MTX concentrations post-infusion in treatment II could be due to slow release of MTX from MTX-RSA conjugate, and could be expected based on in vitro release studies (Fig. 1). It should be noted that the undetectability of MTX in plasma after 8 h post-infusion in Fig. 2 was due to our HPLC sensitivity using the deproteinization method.

Some pharmacokinetic parameters of MTX in treatments I and II are listed in Table 2. It was reported that though albumin is a large molecule, it is not confined to the intravascular space, and

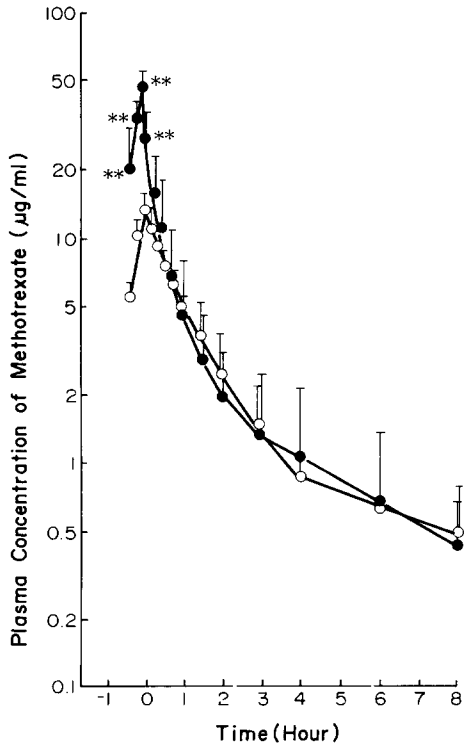

Fig. 2. Mean plasma-level profiles of methotrexate following 30 min infusion of methotrexate, 10 mg/kg (●) and methotrexate-rabbit serum albumin conjugate, 10 mg/kg as methotrexate (○), to rabbits A–D and E–H, respectively. Bars represent standard deviation. * $p < 0.05$, ** $p < 0.01$.

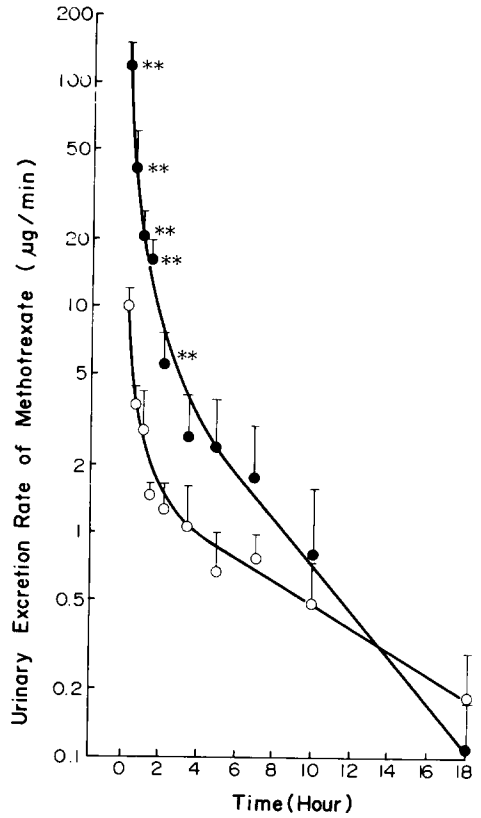

Fig. 3. Mean urinary excretion rates of methotrexate following 30 min infusion of methotrexate, 10 mg/kg (●) and methotrexate-rabbit serum albumin conjugate, 10 mg/kg as methotrexate (○), to rabbits A–D and E–H, respectively. Bars represent standard deviation. * $p < 0.05$, ** $p < 0.01$.

over 30% of total exchangeable albumin which is synthesized in the liver might be in the extravascular space, such as in muscle and skin (Jusko and Gretch, 1976). Moreover, serum albumin has been demonstrated to accumulate at tumor sites (Cerrottini and Isliker, 1967). As shown in Fig. 1, approx. 0.8, 1.4, 3.9, and 8.4% of MTX was released from the conjugate after 6 h of incubation with human plasma, phosphate buffer of pH 7.4, buffer with protease, and rat liver homogenate, respectively. This indicates that small percentages of the attached MTX are physically bound, and that the majority is covalently bound which is slowly released from the conjugate as shown in the MTX-BSA conjugate (Halbert et al., 1987). Therefore, the MTX-RSA conjugate (treatment II) could

be accumulated in tissues, organs, or plasma, and MTX could be released slowly from the conjugate. As a result, the values of V_{SS} and MRT were increased in treatment II (Table 2). Oxidative metabolism of MTX was found to take place in most rabbit tissues, such as liver, lung, small intestine, kidney, heart, and skeletal muscle, with liver showing the greatest activity (Chen and Chiou, 1982). In addition, the formation of 7-hydroxymethotrexate, a metabolite of MTX, after incubation of rabbit blood was very rapid and dose-dependent (Lee et al., 1984). Nonlinear disposition of MTX has also been demonstrated in rabbits (Chen and Chiou, 1983a,b). It suggested that metabolism of slowly released MTX from MTX-RSA conjugate in treatment II could be increased and was supported by a significant increase in CL_{NR} in treatment II. Since the 24 h urinary excretion of MTX was significantly decreased (9.91 vs 1.29 mg) in treatment II as shown in Fig. 4, the CL_R was significantly reduced. The CL showed a trend of increasing ($p < 0.068$) in treatment II due to the decreasing value of AUC (Table 2).

The mean amounts (μg/g tissue) of MTX remaining in each tissue after 30 min of infusion in treatments III and IV are listed in Table 3. The tissue to plasma (T/P) ratios of MTX were below unity for most tissues except kidney after infusion of free MTX (treatment III), and similar results were also reported in rabbit studies (Chen and Chiou, 1982). The kidney, a major eliminating organ for MTX, was found to accumulate large amounts of MTX (105 μg/g kidney) in treatment III. This observation appears to support the possi-

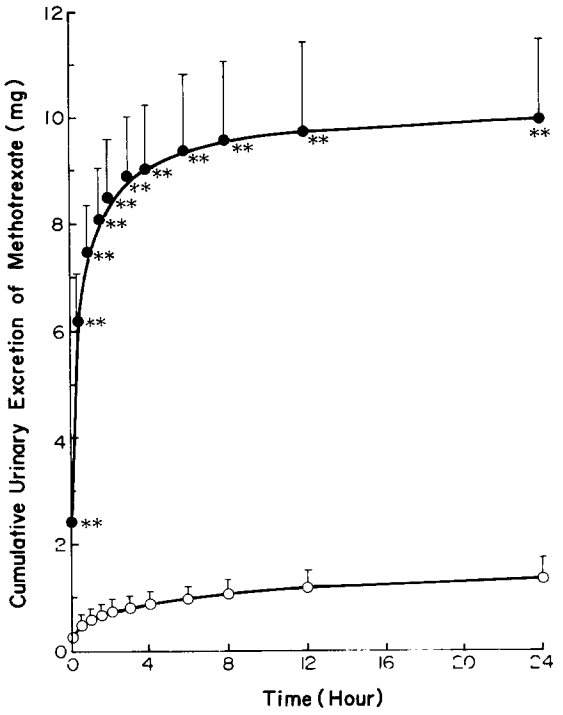

Fig. 4. Mean cumulative amounts of urinary excretion of methotrexate following 30 min infusion of methotrexate, 10 mg/kg (●) and methotrexate-rabbit serum albumin conjugate, 10 mg/kg as methotrexate (○), to rabbits A–D and E–H, respectively. Bars represent standard deviation. * $p < 0.05$, ** $p < 0.01$.

TABLE 2

Mean pharmacokinetic parameters (± S.D.) of methotrexate after 30 min intravenous infusion of methotrexate (treatment I) and methotrexate-rabbit serum albumin conjugate (treatment II) to rabbits A–D, and E–H, respectively

	Treatment I	Treatment II
AUC (μg min ml^{-1})	2360 ± 536 [a]	1510 ± 386
AUMC (μg min^2 ml^{-1})	271000 ± 184000	368000 ± 136000
MRT (h)	1.62 ± 0.991 [a]	3.71 ± 0.701
$t_{1/2}$ (h)	3.26 ± 0.765 [a]	4.96 ± 0.568
V_{SS} (l/kg)	0.311 ± 0.278 [a]	1.470 ± 0.285
CL (ml min^{-1} kg^{-1})	4.25 ± 1.05	6.61 ± 2.00
CL_R (ml min^{-1} kg^{-1})	2.49 ± 0.969 [b]	0.452 ± 0.0481
CL_{NR} (ml min^{-1} kg^{-1})	1.66 ± 0.255 [b]	6.13 ± 1.99
X_u (mg)	9.91 ± 1.82 [b]	1.29 ± 0.404

[a] $p < 0.05$.
[b] $p < 0.01$.

TABLE 3

Mean amounts (± S.D.) of methotrexate remaining in each organ (μg / g tissue) just after 30 min infusion of methotrexate (treatment III) and methotrexate-rabbit serum albumin conjugate (treatment IV) to rabbits I–M, and N–P, respectively

	Treatment III		Treatment IV		Ratio (Treatment IV/ Treatment III)	
	Amount (μg/g tissue)	*T/P* ratio [a]	Amount (μg/g tissue)	*T/P* ratio	Amount ratio	*T/P* ratio
Liver	0.94 ± 0.11	0.0862	3.45 ± 0.36 [d]	1.20	3.67	13.9
Kidney	105 ±29.1	9.64	3.15 ± 0.46 [d]	1.15	0.030	0.113
Stomach	4.36 ± 1.54	0.400	0.72 ± 0.15 [d]	0.250	0.164	0.625
Small intestine	2.36 ± 0.89	0.217	0.76 ± 0.21 [d]	0.264	0.322	1.22
Large intesine	5.72 ± 1.90	0.525	0.56 ± 0.16 [d]	0.194	0.098	0.370
Heart	4.51 ± 2.04	0.414	1.08 ± 0.47 [c]	0.375	0.239	0.906
Muscle	1.89 ± 0.41	0.173	0.73 ± 0.25 [d]	0.253	0.386	1.46
Fat	5.72 ± 1.90	0.525	0.56 ± 0.16 [d]	0.194	0.0979	0.370
Mesentery	3.58 ± 1.49	0.328	1.92 ± 0.27	0.667	0.536	2.03
Lung	5.61 ± 3.08	0.515	1.46 ± 0.36	0.507	0.260	0.984
Spleen	2.12 ± 1.52	0.194	3.32 ± 1.63	1.15	1.57	5.93
Brain	N.D. [b]	–	0.62 ± 1.63 [d]	0.215	–	–
Bile	N.D.	–	N.D.	–	–	–
Plasma	10.9 ±4.91	1.00	2.88 ± 0.576 [c]	1.00	0.264	1.00

[a] Tissue/plasma.
[b] Not detectable.
[c] $p < 0.05$.
[d] $p < 0.01$.

bility that MTX may induce renal failure as it might precipitate in renal tubules during HDMTX therapy (Shen and Azarnoff, 1978). It should be noted that the weight (g/kg body weight) of brain, heart, lung, liver, spleen, stomach or kidney was not significantly different in both treatments.

MTX was significantly concentrated in liver in treatment IV. For example, the ratio of the amounts and the *T/P* ratio in treatment IV were 3.67 and 13.9 times higher, respectively, than those in treatment III (Table 3). The greater uptake of the conjugate by the liver, the main metabolizing organ for MTX in rabbits (Chen and Chiou, 1982), might contribute to increased nonrenal metabolism of MTX in treatment II. However, MTX was significantly less concentrated in kidney and the GI tract after infusion of MTX-RSA conjugate (treatment IV). For example, MTX was approx. 33 times less concentrated in kidney in treatment IV. The corresponding values for stomach, small intestine, and large intestine were 6.1, 3.1, and 10.2. The lower concentrations of MTX in the GI tract and kidney in treatment IV might suggest that the administration of MTX-SA conjugate has lesser side effects in these organs or tissues than those of free MTX. This hypothesis, however, remains to be validated.

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