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Pharmacokinetics of methotrexate after intravenous and intramuscular injection of methotrexate-bearing negatively charged liposomes to rats

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Summary

The pharmacokinetics and tissue distribution of methotrexate (MTX) were investigated after intravenous (i.v.) and intramuscular (i.m.) injection of free MTX (treatment I), freshly prepared MTX-bearing negatively charged liposomes (large unilamellar vesicles), NLUV (treatment II), and empty NLUV mixed manually with free MTX (treatment III), 4 mg kg^{-1} as free MTX to rats using an HPLC assay. After i.v. infusion over 1 min, the plasma concentrations of MTX (C_p), area under the plasma concentration-time curve (AUC, 173 vs 402 μg ml min^{-1}), terminal half-life ($t_{1/2}$, 24.0 vs not determined), mean residence time (MRT, 13.0 vs 83.5 min) and volume of distribution at steady state (V_{ss}, 289 vs 942 ml kg^{-1}) increased significantly, however, total body clearance (CL, 23.1 vs 9.94 ml min^{-1} kg^{-1}), renal clearance (CL_R, 8.38 vs 3.39 ml min^{-1} kg^{-1}), nonrenal clearance (CL_{NR}, 14.6 vs 6.53 ml min^{-1} kg^{-1}) and the amount of MTX excreted in urine (Xu, 415 vs 333 μg) decreased significantly from treatment II when compared with the values from treatment I. This could be due to the fact that some of the MTX-bearing NLUV are entrapped in the tissues, the rest being present in plasma (increase in MRT and V_{ss} from treatment II), and slow release of MTX from MTX-bearing NLUV (increase in $t_{1/2}$ from treatment II). In the present HPLC assay, the concentrations of MTX represent the sum of the free MTX and MTX in MTX-bearing NLUV (increase in C_P and AUC, and decrease in CL from treatment II). Saturable formation of 7-OH-MTX from MTX was observed in rabbit blood, nonlinear disposition of MTX also being found in rabbits (decrease in Xu and CL_R from treatment II). After i.v. infusion over 1 min, some pharmacokinetic parameters of MTX, such as $t_{1/2}$ (24.0 vs 56.9 min), AUC (173 vs 234 μg min ml^{-1}), MRT (13.0 vs 29.8 min), CL (23.1 vs 17.1 ml min^{-1} kg^{-1}), CL_R (8.38 vs 5.66 ml min^{-1} kg^{-1}), CL_{NR} (14.6 vs 11.4 ml min^{-1} kg^{-1}) and Xu (415 vs 290 μg) were significantly different between treatments I and III, however, the differences appeared to be smaller than those between treatments I and II. After both i.v. and i.m. administration, the amount of MTX remaining per g tissue, or the tissue-to-plasma ratio (T/P) of MTX at 30 min after injection was significantly reduced in the kidney, small intestine, large intestine and stomach from treatment II when compared with that from treatment I. This implies that the side effects of MTX on the kidney and GI tract could be reduced after i.v. or i.m. administration of MTX-bearing NLUV when compared with those of free MTX. The encapsulation efficiency of MTX in MTX-bearing NLUV was 4.16% and the MTX was released slowly from MTX-bearing NLUV when incubated in phosphate-buffered saline, rat plasma and rat liver homogenate.

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Introduction

Methotrexate (MTX), a folic acid antagonist, has widely been used in chemotherapy of various types of neoplastic diseases, such as osteosarcoma, choriosarcoma, head-and-neck cancer and breast cancer (Chabner et al., 1975; Bleyer, 1978). MTX exerts its cytotoxic effects by competitively inhibiting dihydrofolate reductase (DHFR), the intracellular enzyme responsible for converting folic acid to reduced folate cofactors (Shen and Azarnoff, 1978; Evans et al., 1986). MTX causes side effects or toxicities on the GI tract (nausea, vomiting, GI desquamation) and kidney (nephrotoxicity), especially at high dose (Shen and Azarnoff, 1978; Evans et al., 1986).

The ideal dosage form in cancer chemotherapy is the one that provides a specific delivery of anticancer agent to the tumor site in a sufficient amount, for a long period of time with no interaction with normal tissue (Yoshioka et al., 1981). For this purpose, liposomes entrapped with anticancer agents provide the possibility of selective drug delivery and reducing systemic cytotoxicity. Factors affecting the passive targeting efficiency such as the localization and distribution of particles in the body are the route of administration, particle size and particle surface characteristics (Torrado et al., 1989). It has been reported that MTX-entrapped negatively charged liposomes enhance the antitumor activity of MTX against CV1-P cells (Heath et al., 1986) when compared with that of free MTX, and that the blood concentrations of total radioactivity are higher (Colley and Ryman, 1975; Claassen and Van Rooijen, 1984) after administration of [^{3}H]MTX-bearing negatively charged liposomes to animals than that of free [^{3}H]MTX. However, most of the studies employed radiolabelled MTX ([^{3}H]MTX)-entrapped negatively charged liposomes (Colley and Ryman, 1975; Claassen and Van Rooijen, 1984), therefore, the measured total radioactivity in biological fluid does not solely represent free MTX, but rather the sum of free MTX, MTX in MTX-bearing negatively charged liposomes and their possible metabolites. MTX has been reported to be metabolized to 7-hydroxymethotrexate (7-OH-MTX), 4-amino-4-deoxy-N^{10}-methylpteroic acid (DAMPA) and MTX-polyglutamate (Shen and Azarnoff, 1978; Evans et al., 1986). 7-OH-MTX and DAMPA were reported to have little affinity for the target enzyme, DHFR (Shen and Azarnoff, 1978; Evans et al., 1986). Moreover, the disposition of 7-OH-MTX was different from that of MTX after intravenous (i.v.) administration of MTX and 7-OH-MTX to rabbits (Chen and Chiou, 1983a,b). Therefore, the measured concentrations of total radioactivity after administration of radiolabelled MTX might not correlate well with the anticancer activity of MTX in terms of binding to DHFR. Hence, in the present study, the concentrations of MTX (not total radioactivity) in plasma, urine and tissue (or organ) were measured after injection of unlabelled free MTX and MTX-bearing negatively charged liposomes to rats.

The purpose of this paper was to determine the pharmacokinetics and tissue distribution of MTX after i.v. and intramuscular (i.m.) administration of unlabelled free MTX and MTX-bearing negatively charged liposomes to rats. The stability of the liposomes was also investigated.

Materials and Methods

Materials

Phosphatidylcholine (PC), cholesterol (CH), α-tocopherol (α-T) and dicetyl phosphate (DCP) were purchased from Sigma Chemical Co. (St. Louis, MO). MTX (a sodium salt for i.v. injection, 500 mg per 20 ml) was kindly supplied by the Choong Wae Pharmaceutical Co. (Suwon, Korea). [3′,5′,7-^{3}H]Methotrexate ([^{3}H]MTX, as a sodium salt) was a product of Amersham International Plc. (Buckinghamshire, U.K.). Other chemicals were reagent grade and used without further purification.

Preparation of liposomes

MTX-bearing negatively charged liposomes (large unilamellar vesicles, LUV), NLUV, were prepared according to a slight modification (10 mg of MTX was used instead of 250 μg of MTX; Han, 1989; Kim et al., 1993) of the reported (Szoka and Papahadjopoulos, 1978) reverse phase

evaporation (REV) method employing lipid molar ratios of PC:CH:DCP:α-T of 8:4:1:0.1. It has been reported (Han, 1989) that the above molar ratio of the lipids is the most appropriate for the preparation of MTX-bearing NLUV. After measurement of the encapsulation efficiency of MTX in MTX-bearing NLUV, the MTX-bearing NLUV were used for animal study within 12 h.

Measurement of encapsulation efficiency

To determine the amount of entrapped MTX in the MTX-bearing NLUV, 50 μl aliquots of MTX-bearing NLUV were first diluted 40 times with phosphate-buffered saline (PBS), and then 400 μl of acetonitrile was added to 50 μl of the diluted MTX-bearing NLUV. After centrifugation, the supernatant was injected directly onto the HPLC column.

Stability test

The stability of free MTX, MTX-bearing NLUV and empty NLUV mixed manually with free MTX was determined in PBS, rat plasma and rat liver homogenate using a dialysis bag as follows (Kim et al., 1993). Each 2.5 ml of PBS, rat plasma and rat liver homogenate (1 g of rat liver was homogenized at 4°C with 4 volumes of PBS and centrifuged at $9000 \times g$ for 10 min, and then the supernatant was used) was added to each clean dialysis bag (Spectra/Por 2 membrane, Mol. Wt cut-off of 12000–14000, Spectrum Medical Ind., Los Angeles, CA) which had been boiled and equilibrated in PBS. Free MTX, MTX-bearing NLUV or empty NLUV mixed manually with free MTX, equivalent to 100 μg as free MTX, was added to each bag. The bag was secured with two knots at each end and the air space minimized as much as possible. Mixing of the contents was performed by squeezing gently and inverting the bag a few times. The bag was washed and wiped dry carefully after trying to ensure that no trace of MTX was found outside the bag. The bag was placed immediately into the dialysate containing 80 ml of PBS kept at 37°C with stirring at a rate of 50 oscillations per min (opm). Timing was started as soon as the bag was immersed in the dialysate. At designated time intervals, 0.05 ml samples were taken from the 80 ml dialysate and stored in a freezer prior to the analysis of MTX.

Pretreatment of rats

76, healthy male Sprague-Dawley (SD) rats (230–330 g, Laboratory Animal Center, Seoul National University, Seoul, Korea) were employed in this study. The carotid artery and jugular vein were cannulated with polyethylene tubing (PE-60, Clay Adams, Parsippany, NJ) under light ether anesthesia. Both cannulas were exteriorized to the dorsal side of the neck, where each cannula terminated with long silastic tubing (Dow Corning Co., Midland, MI). The silastic tubings were covered with wire to allow free movement of the rat. The exposed areas were closed using a surgical suture. Each rat was housed in a rat metabolic cage (Daejong Scientific Co., Seoul, Korea), and allowed to recover from anesthesia for 4–5 h before study.

Intravenous infusion studies

Free MTX (i.v. solution as a sodium salt, 500 mg per 20 ml, diluted with 0.9% NaCl injectable solution to make 1 mg ml^{-1}; treatment I, $n = 8$), freshly prepared MTX-bearing NLUV (treatment II, $n = 6$) or empty NLUV mixed manually with free MTX (treatment III, $n = 5$), equivalent to 4 mg kg^{-1} as free MTX were administered via the jugular vein by i.v. infusion over a period of 1 min (injection volume approx. 1 ml) to rat. Blood samples (0.12 ml) were collected via the carotid artery at 0 (to serve as a control), 1 (at the end of infusion), 5, 15, 30, 45, 60, 90, 120, 180 and 240 min for treatments I and III, and at 0, 1, 5, 15, 30, 45, 60, 90, 120, 180, 240, 360, 480, 600, 720, 960 and 1440 min for treatment II after i.v. injection. Heparinized normal saline solution (10 U ml^{-1}), 0.25 ml was used for flushing the cannula just after each blood sampling to prevent blood from clotting. The blood samples were centrifuged immediately to reduce the possible 'blood storage effect' of the plasma concentrations of MTX (Lee et al., 1984, 1986), and 50 μl of plasma was stored in the freezer prior to measurement of MTX. At the end of 24 h (for treatments I and III), and 24 and 48 h (for treatment II) after i.v. injection, the metabolic cage was rinsed with 20 ml of distilled

water and the rinsings were combined with urine. After measuring the exact volume of the combined urine, two 0.1 ml portions of the combined urine were frozen prior to analysis for MTX.

Intramuscular injection studies

The carotid artery of rat was similarly cannulated with polyethylene tubing under light ether anesthesia. Free MTX (treatment IV, $n = 6$), freshly prepared MTX-bearing NLUV (treatment V, $n = 5$) or empty NLUV mixed manually with free MTX (treatment VI, $n = 7$), equivalent to 4 mg kg^{-1} as free MTX were injected (injection volume approx. 0.3 ml) into the center of the right thigh muscle (musculus rectus) of the rat. Blood samples (0.12 ml) were collected via the carotid artery at 0, 15, 30, 45, 60, 90, 120, 180 and 240 min for treatments IV and VI, and at 0, 15, 30, 45, 60, 90, 120, 180, 240, 360, 480, 600 and 720 min for treatment V after i.m. injection. The blood and urine samples were handled similarly to the i.v. infusion studies. Free MTX ($n = 3$), freshly prepared MTX-bearing NLUV ($n = 3$) or empty NLUV mixed manually with free MTX ($n = 3$), equivalent to 4 mg kg^{-1} as free MTX was similarly injected intramuscularly. At the end of 8 h, as much of the thigh muscle of the injection site as possible was removed, homogenized with 4 volumes of 0.02 N NaOH, and then vortex-centrifuged. Two 0.1 ml portions of the supernatant were stored in the freezer prior to HPLC assay of MTX.

Tissue distribution studies after i.v. infusion

MTX (treatment VII, $n = 5$), freshly prepared MTX-bearing NLUV (treatment VIII, $n = 5$) and empty NLUV mixed manually with free MTX (treatment IX, $n = 5$), equivalent to 4 mg kg^{-1} as free MTX were similarly infused over 1 min via the jugular vein of rats. After 30 min from the start of infusion, as much blood as possible was collected through the carotid artery and each rat was exsanguinated. After centrifugation of the blood, plasma was added with 4 volumes of 0.02 N NaOH and vortex-centrifuged. Approx. 1 g of the heart, lung, spleen, brain, liver, kidney, stomach, small intestine, large intestine and thigh muscle was quickly removed, rinsed, minced, homogenized with 4 volumes of 0.02 N NaOH in a tissue homogenizer (Ultra-Turrax T 25, Janke & Kunkel, IKA-Labortechnik, Germany), and then centrifuged immediately. Two 0.1 ml aliquots of plasma or supernatant of tissue homogenates were stored in the freezer prior to MTX analysis. The ipsilateral iliac lymph nodes were also removed, rinsed, minced and allowed to stand overnight at room temperature with 4 volumes of 0.2 N NaOH. After centrifugation, as much supernatant as possible was sampled and analyzed for MTX.

Tissue distribution studies after i.m. injection

Free MTX (treatment X, $n = 5$), freshly prepared MTX-bearing NLUV (treatment XI, $n = 5$) and empty NLUV mixed manually with free MTX (treatment XII, $n = 5$), equivalent to 4 mg kg^{-1} as free MTX, were similarly injected intramuscularly to rats. Other procedures were similar to those of the tissue distribution studies after i.v. infusion.

Pharmacokinetic analysis

The area under the plasma concentration-time curve from time zero to time infinity (AUC) was calculated according to the trapezoidal rule-extrapolation method (Yoon et al., 1991); this method employed the logarithmic trapezoidal rule for the calculation of area during the declining plasma-level phase (Chiou, 1978) and the linear trapezoidal rule for the rising plasma-level phase. The area from the last data point to time infinity was estimated by dividing the last measured plasma concentration by the terminal rate constant.

A standard method (Gibaldi and Perrier, 1982) was used to calculate the following pharmacokinetic parameters: time-averaged total body clearance (CL), area under the first moment of the plasma concentration-time curve (AUMC), mean residence time (MRT), apparent volume of distribution at steady state (V_{ss}), and time-averaged renal (CL_R) and nonrenal (CL_{NR}) clearances:

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

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

$$MRT = AUMC/AUC \quad (3)$$

$$V_{ss} = CL \cdot MRT \quad (4)$$

$$CL_R = Xu/AUC \quad (5)$$

$$CL_{NR} = CL - CL_R \quad (6)$$

where C_p is the plasma concentration of MTX at time t, and Xu denotes the amount of MTX excreted in urine up to time infinity (this was assumed to equal the total amount of MTX excreted in 24 and 48 h for treatments I and III, and treatment II, respectively, since only a negligible amount of MTX could be found in the urine collected later). Only AUC and CL_R were measured after i.m. administration.

The percentages of the dose absorbed into the general circulation after i.m. administration (F) were determined based on the following plasma area-clearance method (Chiou et al., 1981; Lee and Chiou, 1983):

$$F = 100(CL_{i.m.} \cdot AUC_{i.m.})/dose_{i.m.} \quad (7)$$

where $CL_{i.m.}$, the CL after i.m. administration was calculated by the summation of CL_R obtained from the i.m. study ($CL_{R,i.m.}$) and CL_{NR} obtained from the i.v. study ($CL_{NR,i.v.}$) as illustrated below:

$$CL_{NR} = CL_{i.v.} - CL_{R,i.v.} \quad (8)$$

$$CL_{i.m.} = CL_{NR} + CL_{R,i.m.} \quad (9)$$

The mean values of each clearance, V_{ss} and half-life were calculated using the harmonic mean method (Chiou, 1979).

HPLC analysis

The concentrations of MTX in plasma, urine and tissues (or organs) were determined employing a slight modification of the mobile phase of the reported HPLC method (Chen and Chiou, 1981; Lee et al., 1984). The HPLC system consisted of a Model 7125 injector (Rheodyne, Cotati, CA), a Model 400 solvent delivery system pump (Applied Biosystems, San Jose, CA), two cationic exchange columns, a guard column (CX-300, 30 × 2.1 mm, Applied Biosystems) and an analytical column (SCX, 25 cm × 2.9 mm i.d., 10 μm, Whatman, Clifton, NJ), a Model 1306 UV detector (Bio-Rad, Japan Servo Co., Japan) and a Model 1200 recorder (Linear, Reno, NV). The mobile phase, 0.02 M $NH_4H_2PO_4$-acetonitrile (9:1, v/v) adjusted to pH 1.6 with phosphoric acid, was run at a flow rate of 2.0 ml min^{-1} (resultant pressure approx. 1300 $lb/inch^2$) and the eluent was detected at 313 nm. Biological samples were deproteinized with 2.5 volumes of acetonitrile. After vortex-mixing and centrifugation, 50 μl of the supernatant were injected onto the column. Peak height measurements were used for quantitation of MTX.

Statistical analysis

Levels of statistical significance were assessed using the analysis of variance (ANOVA) test between the two means for unpaired data. Significant differences were judged as a p value of less than 0.05. All results are expressed as mean ± standard deviation.

Results and Discussion

Measurement of encapsulation efficiency

The encapsulation efficiency of MTX in MTX-bearing NLUV prepared by the REV method was examined at various molar ratios of lipids (Han, 1989); the efficiency increased with increasing CH up to 33 mol%. CH is known to reduce the permeability of a drug from liposomes, therefore, it ensures the stability of liposomes (Allen and Cleland, 1980). The mean encapsulation efficiency was 4.16 ± 1.54% ($n = 4$). In order to compare encapsulation efficiency, [^{3}H]MTX-bearing NLUV were also prepared and the efficiency was measured using both total radioactivity assay (Kim et al., 1993) and the HPLC method; the values were found to be 3.79 and 2.90% for the HPLC method and total radioactivity assay, respectively. Similar efficiencies for the HPLC method and total radioactivity assay have also been reported for MTX-bearing positively charged liposomes, i.e., 4.15 vs 3.50% (Kim, 1993) and MTX-bearing neutral liposomes, 3.92 vs 3.60% (Bae et al., 1994).

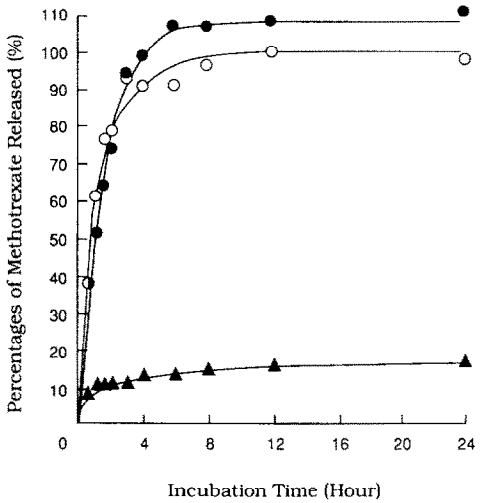

Fig. 1. Percentages of methotrexate (MTX) released from the semipermeable bag containing free MTX (●), MTX-bearing negatively charged liposomes (▲) and empty negatively charged liposomes mixed manually with free MTX (○) which were incubated in PBS, kept at 37°C and at a rate of 50 opm as a function of time.

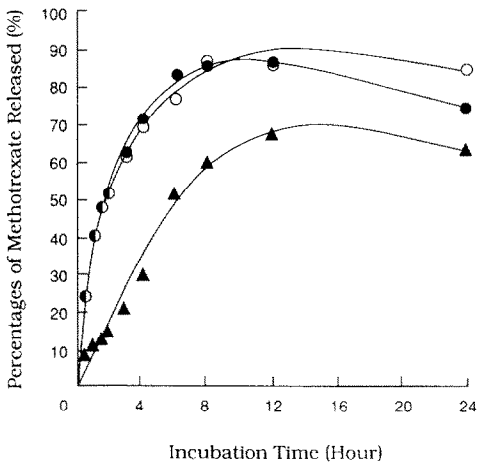

Fig. 3. Percentages of methotrexate (MTX) released from the semipermeable bag containing free MTX (●), MTX-bearing negatively charged liposomes (▲) and empty negatively charged liposomes mixed manually with free MTX (○) which were incubated in rat liver homogenate, kept at 37°C and at a rate of 50 opm as a function of time.

In vitro release of methotrexate

Figs 1–3 show the percentages of MTX released from the semipermeable bag containing free MTX, MTX-bearing NLUV and empty NLUV mixed manually with free MTX which were incubated in PBS, rat plasma and rat liver homogenate, respectively, kept at 37°C and at a rate of 50 opm as a function of time. MTX was released essentially completely from free MTX for up to 6–8 h of incubation in both PBS (Fig. 1) and rat plasma (Fig. 2). However, the corresponding value decreased in the rat liver homogenate (Fig. 3) due to the formation of a metabolite, which was supported by the increase in the peak of the unknown metabolite (the peak appearing just before that of MTX) with increasing incubation time in the HPLC chromatogram. MTX is known to be metabolized in humans (Shen and Azarnoff, 1978; Evans et al., 1986) and in tissues or organs (Chen and Chiou, 1982) and blood (Lee et al., 1984) from rabbits. The release of MTX from empty NLUV mixed manually with free MTX appeared to be comparable to that of free MTX in all of the incubation media studied. In contrast, the release of MTX from MTX-bearing NLUV was considerably lower than that of free MTX or empty NLUV mixed manually with free MTX in all of the incubation media examined; the percentages of MTX released from the

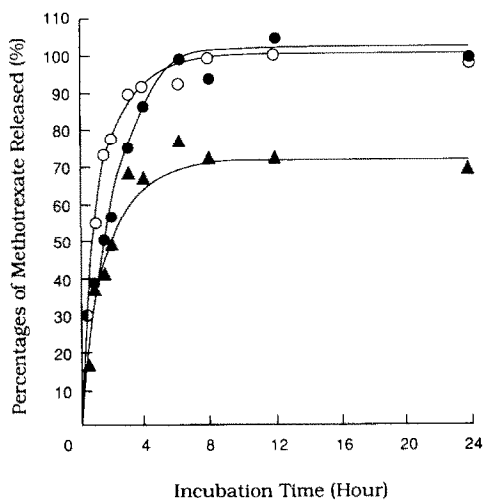

Fig. 2. Percentages of methotrexate (MTX) released from the semipermeable bag containing free MTX (●), MTX-bearing negatively charged liposomes (▲) and empty negatively charged liposomes mixed manually with free MTX (○) which were incubated in rat plasma, kept at 37°C and at a rate of 50 opm as a function of time.

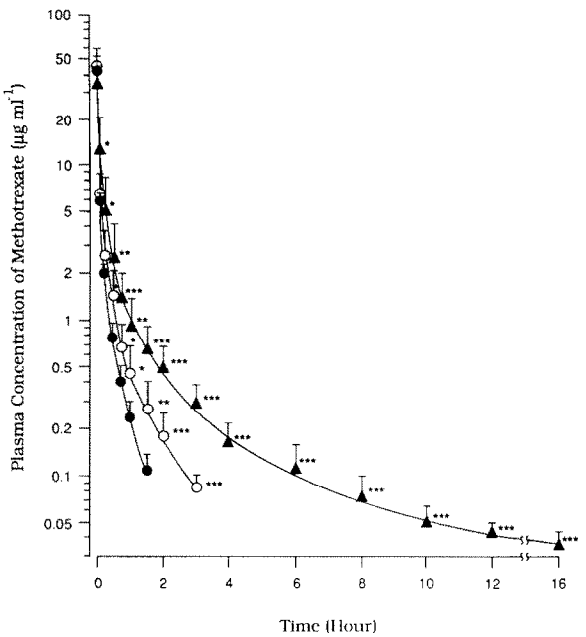

Fig. 4. Mean arterial plasma concentration-time profiles of methotrexate (MTX) after 1 min intravenous infusion of free MTX (treatment I, $n = 8$, ●), MTX-bearing negatively charged liposomes (treatment II, $n = 6$, ▲) and empty negatively charged liposomes mixed manually with free MTX (treatment III, $n = 5$, ○), 4 mg kg^{-1} as free MTX to rats. Bars represent standard deviation. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ when compared with the values from treatment I.

semipermeable bag up to 24 h of incubation were 16.2, 69.3 and 64.2% when MTX-bearing NLUV were incubated in PBS (Fig. 1), rat plasma (Fig. 2) and rat liver homogenate (Fig. 3), respectively.

Pharmacokinetics of MTX after i.v. administration

Fig. 4 shows the mean arterial plasma concentration-time profiles of MTX from treatments I–III, and the relevant pharmacokinetic parameters of MTX are listed in Table 1. After i.v. infusion, the plasma concentrations of MTX declined polyexponentially in all of the rats studied and the levels decayed rapidly from treatments I and III with mean terminal half-lives of 24.0 and 56.9 min, respectively. From treatment II, the terminal half-life could not be determined, since the terminal phase in plasma concentrations of MTX could not be achieved (Fig. 1). However, the plasma concentrations from treatment II were significantly higher than those from treatment I and declined slowly in the terminal phase. Previously, it was reported that blood total radioactivity was higher when [^{3}H]MTX-bearing negatively charged liposomes were administered intravenously to rats (Colley and Ryman, 1975) and mice (Claassen and Van Rooijen, 1984) than that

TABLE 1

Mean (±standard deviation) pharmacokinetic parameters of methotrexate (MTX) after 1 min intravenous infusion of free MTX (treatment I), MTX-bearing negatively charged liposomes (treatment II) and empty negatively charged liposomes mixed manually with free MTX (treatment III), 4 mg kg^{-1} to rats

Parameters	Treatment I ($n = 8$)	Treatment II ($n = 6$)	Treatment III ($n = 5$)
Body weight (g)	284 ± 13.6	246 ± 19.6	220 ± 15.4
$t_{1/2}$ (min)	24.0 ± 2.11	N.D.	56.9 ± 35.9 [a]
AUC (μg min ml^{-1})	173 ± 16.9	402 ± 175 [b]	234 ± 37.1 [b]
AUMC (μg min^2 ml^{-1})	2 240 ± 476	35 300 ± 23 500 [b]	6 790 ± 1 160 [c]
MRT (min)	13.0 ± 2.71	83.5 ± 44.8 [c]	29.8 ± 7.65 [c]
V_{ss} (ml kg^{-1})	289 ± 74.5	942 ± 504 [b]	474 ± 233
CL (ml min^{-1} kg^{-1})	23.1 ± 2.21	9.94 ± 4.78 [c]	17.1 ± 2.76 [b]
CL_R (ml min^{-1} kg^{-1})	8.38 ± 0.860	3.39 ± 1.55 [c]	5.66 ± 0.630 [c]
CL_{NR} (ml min^{-1} kg^{-1})	14.6 ± 2.11	6.53 ± 3.25 [c]	11.4 ± 2.55 [c]
Xu (μg)	415 ± 53.2 [d]	333 ± 30.6 [b,e]	290 ± 27.6 [c,d]

[a] $p < 0.05$, [b] $p < 0.01$ and [c] $p < 0.001$ when compared with the values from treatment I. N.D., not determined because the terminal phase could not be obtained. [d] 0–24 h; [e] 0–48 h.

of free [^{3}H]MTX. The slow decay of terminal plasma MTX from treatment II could be due to the slow release of MTX from MTX-bearing NLUV which are entrapped in tissues (discussed below in the section, Tissue distribution study; Table 3) or present in plasma. The slow decay of plasma MTX from treatment II was expected from the in vitro release study (Figs 1–3). Plasma concentrations of MTX were detected for up to 1.5, 16 and 3 h after i.v. administration from treatments I–III, respectively, and could be due to our assay sensitivity using the deproteinization method (Chen and Chiou, 1981; Lee et al., 1984).

Since the apparent terminal phase in plasma concentrations of MTX was not attained for up to 16 h after i.v. administration in all of the rats studied from treatment II, the pharmacokinetic data were estimated based on the plasma concentrations up to 16 h. MTX was not detected in plasma and urine samples after 3 and 24 h, respectively, from treatments I and III. Therefore, the pharmacokinetic parameters from treatments I and III which are listed in Table 1 (based on plasma concentrations of MTX up to time infinity) could be very close to those based on the data for plasma concentrations up to 16 h (mean contributions of the AUC from 16 h to time infinity to the total AUC were 2.67×10^{-11} and 2.22×10^{-4}% from treatments I and III, respectively). The MTX entrapped in MTX-bearing NLUV may be neither excreted via the kidney nor metabolized, and MTX is slowly released from MTX-bearing NLUV which are entrapped in tissues or present in the plasma. In the present HPLC assay, the concentrations of MTX in plasma represent both free MTX and MTX entrapped in MTX-bearing NLUV. Therefore, the plasma concentrations of MTX from treatment II were significantly higher than those from treatment I (Fig. 4), and resulted in a significant increase in AUC (173 vs 402 μg min ml^{-1}) from treatment II. As expected, CL decreased significantly (23.1 vs 9.94 ml min^{-1} kg^{-1}) from treatment II. As stated earlier, some of the MTX-bearing NLUV are entrapped in tissues the remainder being present in plasma, and MTX is released slowly from MTX-bearing NLUV, therefore, MRT (13.0 vs 83.5 min) and V_{ss} (289 vs 942 ml kg^{-1}) increased significantly from treatment II when compared with those from treatment I.

The saturable formation of 7-OH-MTX from MTX has been reported in rabbit blood (Lee et al., 1984). Moreover, oxidative metabolism of MTX has been found to take place in most rabbit tissues, such as the liver, lung, stomach, kidney, heart and skeletal muscle, with the liver showing the greatest activity (Chen and Chiou, 1982). Nonlinear disposition of MTX has also been reported in rabbits (Chen and Chiou, 1983a,b). Therefore, the free MTX released slowly from MTX-bearing NLUV which are entrapped in tissues or present in plasma (treatment II) could be metabolized more rapidly than that from treatment I. This was demonstrated by the significant decrease in Xu (415 vs 333 μg) and the resultant decrease in CL_R (8.38 vs 3.39 ml min^{-1} kg^{-1}) from treatment II when compared with that from treatment I. Similar results were also reported previously (Yoon et al., 1991), showing that the Xu was significantly reduced when MTX-rabbit serum albumin conjugates (some of the conjugates were entrapped in tissues, the rest being present in plasma, and MTX was released slowly from the conjugates) were intravenously administered to rabbits when compared with that of free MTX. The significant decrease in CL from treatment II could be due to a significant decrease in both CL_R (8.38 vs 3.39 ml min^{-1} kg^{-1}) and CL_{NR} (14.6 vs 6.53 ml min^{-1} kg^{-1}). It should be noted that some of the pharmacokinetic parameters of MTX could be affected by i.v. administration of empty NLUV (treatment III); the values of $t_{1/2}$ (24.0 vs 56.9 min), AUC (173 vs 234 μg min ml^{-1}), MRT (13.0 vs 29.8 min), CL (23.1 vs 17.1 ml min^{-1} kg^{-1}), CL_R (8.38 vs 5.66 ml min^{-1} kg^{-1}), CL_{NR} (14.6 vs 11.4 ml min^{-1} kg^{-1}) and Xu (415 vs 290 μg) were significantly different between treatments I and III, however, the differences appeared to be smaller than those between treatments I and II.

Pharmacokinetics of MTX after i.m. administration

Fig. 5 shows the mean arterial plasma concentration-time profiles of MTX from treatments IV–VI and the corresponding pharmacokinetic

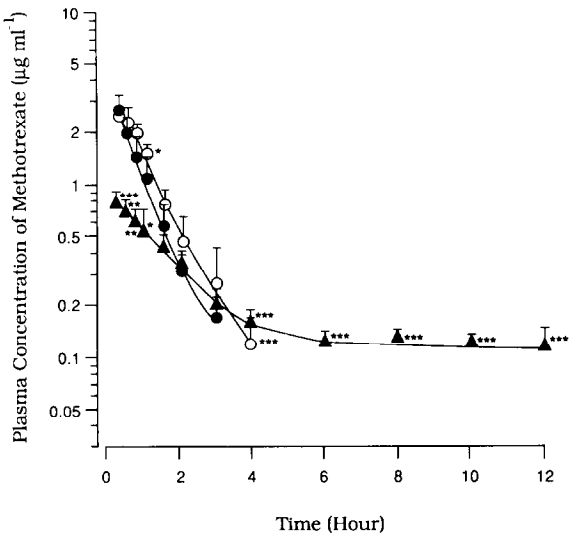

Fig. 5. Mean arterial plasma concentration-time profiles of methotrexate (MTX) after intramuscular injection of free MTX (treatment IV, $n = 6$, ●), MTX-bearing negatively charged liposomes (treatment V, $n = 5$, ▲) and empty negatively charged liposomes mixed manually with free MTX (treatment VI, $n = 7$, ○), 4 mg kg^{-1} as free MTX to rats. Bars represent standard deviation. * $p < 0.5$, ** $p < 0.01$ and *** $p < 0.001$ when compared with the values from treatment IV.

data are listed in Table 2. The absorption of MTX after i.m. injection was rapid and almost complete; the maximum plasma concentration of MTX was reached at 15 min after i.m. injection from treatments IV and VI. The extent of bioavailability (F) after i.m. administration of free MTX (treatment IV) was estimated to be 81.4%, the corresponding value for empty NLUV mixed manually with free MTX (treatment VI) being 92.2%. The rapid and almost complete absorption of MTX from treatments IV and VI could be supported by the fact that the percentages of the i.m. dose remaining at the injection site at 8 h after i.m. administration were negligible; the values were determined as 0.567 ± 0.239 and 0.278 ± 0.0982% when free MTX ($n = 3$) and empty NLUV mixed manually with free MTX ($n = 3$), respectively, were injected i.m. to other rats. The rapid and almost complete absorption of MTX after i.m. administration of free MTX has also been reported in rabbits (Yoon et al., 1990) and humans (Shen and Azarnoff, 1978).

In contrast to the results after i.v. administration, the plasma concentrations of MTX declined monoexponentially from treatments IV and VI with mean terminal half-lives of 37.2 and 56.6 min, respectively. After treatment V, the mean plasma concentrations of MTX at 15 min were significantly lower (0.794 vs 2.66 and 2.49 μg ml^{-1}) than those from treatments IV and VI, and the plasma concentrations seemed to remain almost constant from 4 to 12 h. Therefore, the terminal half-life could not be determined, and hence in the estimation of CL_R, the plasma concentrations up to 12 h were employed. The lower plasma concentrations of MTX in the initial phase and the slow decay of plasma concentrations of MTX from treatment V could be due to the slow absorption of MTX from the injection site. The slow absorption of MTX from the injection site

TABLE 2

Mean (±standard deviation) pharmacokinetic parameters of methotrexate (MTX) after intramuscular injection (right thigh muscle) of free MTX (treatment IV), MTX-bearing negatively charged liposomes (treatment V) and empty negatively charged liposomes mixed manually with free MTX (treatment VI), 4 mg kg^{-1} as free MTX to rats

Parameters	Treatment IV ($n = 6$)	Treatment V ($n = 5$)	Treatment VI ($n = 7$)
Body weight (g)	307 ± 22.3	239 ± 13.4	251 ± 24.2
$t_{1/2}$ (min)	37.2 ± 13.3	N.D.	56.6 ± 16.7 [a]
AUC (μg min ml^{-1})	158 ± 34.2	152 ± 21.7	205 ± 39.3 [a]
CL_R (ml min^{-1} kg^{-1})	5.99 ± 2.62	7.83 ± 2.44	6.60 ± 1.21
Xu (μg)	319 ± 86.8 [b]	292 ± 36.4 [c]	343 ± 79.2 [b]

[a] $p < 0.05$ when compared with the values from treatment IV. N.D., not determined because the terminal phase could not be obtained. [b] 0–24 h; [c] 0–48 h.

after treatment V could be supported by the fact that the percentages of the dose remaining at the injection site at 8 h after i.m. administration were considerable, the value being 51.5 ± 3.93%. It has been reported (Stevenson et al., 1982; Patel et al., 1984) that, when injected subcutaneously, liposomes are drained from the site of injection into the circulation via the lymphatics; in the lymphatics, some liposomes are degraded, some are retained in the lymph nodes and the rest reach the circulation. It could be expected that the MTX present in the systemic circulation after treatment V could be absorbed MTX, i.e., as free MTX which was absorbed from the injection site and MTX absorbed from the lymphatics. As explained in the i.v. study, the Xu from treatment V (292 μg) was expected to be significantly lower than that from treatment IV (319 μg). However, the values of Xu ($p < 0.5403$) were not significantly different between treatments IV and V. The lack of significance between these values may reflect the limited number ($n = 5$–6) of rats studied and/or might be due to intersubject variation between the rats. The effect of empty NLUV (treatment VI) on the pharmacokinetic parameters of MTX was significant in terms of $t_{1/2}$ (37.2 vs 56.6 min) and AUC (158 vs 205 μg min ml^{-1}).

Tissue distribution of MTX after i.v. administration

The mean amount (μg per g tissue) of MTX remaining per g tissue (or organ), and tissue to plasma ratio (T/P) from treatments VII–IX are listed in Table 3. MTX was highly concentrated in the kidney, small intestine, large intestine, stomach, muscle and liver (34.7, 105, 17.8, 11.2, 7.87 and 23.9 μg per g tissue) as reflected in the T/P ratios of greater than unity (14.6, 39.1, 6.79, 4.38, 3.23 and 9.19) from treatment VII, and the results differ from those in rabbits in which only the kidney showed a T/P ratio above unity (Chen and Chiou, 1982; Yoon et al., 1991). The T/P value was extremely high in the kidney, small intestine, large intestine and stomach from treatment VII and might explain the GI and kidney toxicity after administration of MTX. However, the T/P values of the kidney (14.6 vs 1.11), small intestine (39.1 vs 0.782), large intestine (6.79 vs 0.0970) and stomach (4.38 vs 0.116) were significantly reduced from treatment VIII when compared with those from treatment VII. The above data might suggest that the i.v. administration of MTX-bearing NLUV has lesser side effects in these tissues (or organs) than those of free MTX, however, this hypothesis remains to be validated. The amount of MTX remaining per g spleen

TABLE 3

Mean (±standard deviation) amount (μg per g tissue) of methotrexate (MTX) remaining in g tissue or organ at 30 min after intravenous infusion of free MTX (treatment VII), MTX-bearing negatively charged liposomes (treatment VIII) and empty negatively charged liposomes mixed manually with free MTX (treatment IX), 4 mg kg^{-1} to rats

Tissues	Treatment VII ($n = 5$)	Treatment VIII ($n = 5$)	Treatment IX ($n = 5$)
Plasma	2.61 ± 0.493	6.65 ± 4.33	3.01 ± 2.67
Spleen	1.54 ± 0.252 (0.650 ± 0.174)	111 ± 35.9 [c] (21.5 ± 9.94) [b]	0.785 ± 0.266 [b] (0.409 ± 0.238)
Kidney	34.7 ± 7.24 (14.6 ± 4.26)	5.29 ± 3.37 [c] (1.11 ± 0.767) [c]	6.66 ± 1.28 [c] (3.03 ± 1.21) [c]
Small intestine	105 ± 65.6 (39.1 ± 23.5)	2.83 ± 2.72 [a] (0.782 ± 0.760) [a]	21.9 ± 17.3 [a] (12.2 ± 10.6) [a]
Large intestine	17.8 ± 10.6 (6.79 ± 3.71)	0.440 ± 0.0894 [b] (0.0970 ± 0.0682) [b]	0.548 ± 0.240 [b] (0.282 ± 0.206) [b]
Stomach	11.2 ± 1.84 (4.38 ± 0.948)	0.503 ± 0.0974 [c] (0.116 ± 0.0980) [c]	0.486 ± 0.0478 [c] (0.234 ± 0.111) [c]
Lung	1.31 ± 0.520 (0.505 ± 0.186)	9.83 ± 4.16 [b] (2.04 ± 1.27) [a]	0.591 ± 0.187 [a] (0.270 ± 0.141)
Heart	0.904 ± 0.709 (0.374 ± 0.318)	1.58 ± 0.593 (0.305 ± 0.160)	0.381 ± 0.283 (0.149 ± 0.136)
Right thigh muscle	7.87 ± 2.39 (3.23 ± 1.44)	0.283 ± 0.0588 [c] (0.0640 ± 0.0496) [b]	0.565 ± 0.106 [c] (0.273 ± 0.137) [b]
Liver	23.9 ± 6.41 (9.19 ± 1.65)	10.8 ± 4.15 [b] (2.77 ± 3.04) [c]	3.67 ± 0.680 [c] (1.75 ± 0.754) [c]
Lymph nodes	1.35 ± 0.269 (0.528 ± 0.135)	0.445 ± 0.0376 [c] (0.0642 ± 0.0230) [c]	0.796 ± 0.228 [b] (0.468 ± 0.0590)
Brain	1.49 ± 0.279 (0.601 ± 0.220)	0.362 ± 0.119 [c] (0.0754 ± 0.0536) [c]	U.D. [c]

Values in parentheses represent mean (±standard deviation) values of the tissue-to-plasma ratio. [a] $p < 0.05$, [b] $p < 0.01$ and [c] $p < 0.001$ when compared with the values from treatment VII. U.D., under detection limit.

(1.54 vs 111 μg) and T/P ratio (0.650 vs 21.5) increased significantly from treatment VIII when compared with those from treatment VII. Therefore, i.v. administration of MTX-bearing NLUV might have targeting ability to the spleen. Similar results were also obtained when [^{3}H]MTX bearing negatively charged liposomes were administered intramuscularly to mice (Claassen and Van Rooijen, 1984) and rats (Colley and Ryman, 1975). It should be noted that the tissue distribution of MTX could be somewhat affected by i.v. administration of empty NLUV (treatment IX); the T/P ratios were significantly different between treatments VII and IX in all the tissues or organs studied except the spleen, lung and heart, however, the differences appeared to be smaller than those between treatments VII and VIII.

Tissue distribution of MTX after i.m. administration

The mean amount (μg per g tissue) of MTX remaining per g tissue (or organ) and tissue to plasma ratio (T/P) from treatments X–XII are listed in Table 4. The results differ from those after i.v. administration; the T/P ratios of MTX were higher than unity for the kidney, small intestine, liver and lymph nodes (3.55, 19.5, 1.64 and 1.20) from treatment X. It interesting to note that from treatment XI, the amount of MTX remaining per g tissue (or organ) at 30 min after i.m. administration in the plasma (3.68 vs 1.36 μg), kidney (12.1 vs 3.72 μg), small intestine (64.5 vs 3.13 μg) and liver (6.03 vs 2.28 μg) was significantly lower than that in treatment X. The tissue distribution of MTX was also affected by i.m. administration of empty NLUV (treatment XII) such as the spleen, stomach, lung, heart, lymph nodes and brain.

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TABLE 4

Mean (±standard deviation) amount (μg per g tissue) of methotrexate (MTX) remaining in g tissue or organ at 30 min after intramuscular injection (right thigh muscle) of free MTX (treatment X), MTX-bearing negatively charged liposomes (treatment XI) and empty negatively charged liposomes mixed manually with free MTX (treatment XII), 4 mg kg^{-1} to rats

Tissues	Treatment X ($n = 5$)	Treatment XI ($n = 5$)	Treatment XII ($n = 5$)
Plasma	3.68 ± 0.494	1.36 ± 0.175 [c]	4.59 ± 1.18
Spleen	0.328 ± 0.0516 (0.0933 ± 0.0142)	0.686 ± 0.339 [a] (0.481 ± 0.208) [b]	0.526 ± 0.0930 [b] (0.118 ± 0.0214)
Kidney	12.1 ± 6.28 (3.55 ± 2.43)	3.72 ± 0.542 [a] (2.87 ± 0.515)	12.0 ± 1.13 (2.74 ± 0.615)
Small intestine	64.5 ± 53.0 (19.5 ± 19.9)	3.13 ± 1.80 [a] (2.28 ± 1.38)	12.4 ± 13.5 (2.56 ± 2.59)
Large intestine	0.844 ± 0.169 (0.230 ± 0.0338)	0.694 ± 0.227 (0.513 ± 0.197) [a]	1.11 ± 0.582 (0.241 ± 0.104)
Stomach	0.555 ± 0.0993 (0.155 ± 0.0452)	1.26 ± 0.976 (0.987 ± 0.784) [a]	0.889 ± 0.130 [b] (0.195 ± 0.0800)
Lung	0.779 ± 0.175 (0.218 ± 0.0759)	0.774 ± 0.265 (0.580 ± 0.152)	1.34 ± 0.136 [c] (0.304 ± 0.0602)
Heart	0.552 ± 0.174 (0.151 ± 0.0476)	0.516 ± 0.0926 (0.367 ± 0.0198) [b]	1.12 ± 0.163 [a] (0.251 ± 0.0644)
Liver	6.03 ± 2.45 (1.64 ± 0.645)	2.28 ± 0.325 [a] (1.66 ± 0.109)	5.36 ± 1.04 (1.23 ± 0.413)
Lymph nodes	4.40 ± 1.35 (1.20 ± 0.273)	5.66 ± 2.73 (4.06 ± 1.53) [b]	7.26 ± 1.64 [a] (1.72 ± 0.735)
Brain	U.D.	0.544 ± 0.0790 [c] (0.392 ± 0.113) [c]	0.321 ± 0.111 [c] (0.0736 ± 0.0338) [b]

Values in parentheses represent mean (±standard deviation) values of the tissue-to-plasma ratio. [a] $p < 0.05$, [b] $p < 0.01$ and [c] $p < 0.001$ when compared with the values from treatment X. U.D., under detection limit.

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