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Non-Michaelis–Menten Type Hepatic Uptake of Liposomes in the Rat

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Abstract—The objective of this study was to verify the methodology for measuring uptake clearance of liposomes and to characterize kinetically the saturable hepatic uptake of liposomes through phagocytosis. The correction of vascular space was important in the evaluationof hepatic uptake. The efflux of liposomes from liver was shown to be negligible, by a repeated dose study, and thus, hepatic clearance can be obtained by the hepatic uptake divided by the area under the blood concentration-time curve (AUC). The determinant parameter which describes the saturability of uptake clearance of liposomes, independent of infusion rate, was investigated, using the data of an in-vivo constant infusion study, where infusion rate-dependent saturable hepatic clearance was observed. The mean blood concentration failed to obtain an infusion rate-independent function. On the other hand, the AUC could explain the saturability of hepatic clearance for every infusion rate by a unique relationship. The hepatic uptake amount could also explain this saturability, independent of infusion rate. These kinetic characteristics are inconsistent with Michaelis–Menten type kinetics, therefore a new model is required to describe the saturable hepatic clearance in the disposition of liposomes.

Developments in biotechnology have allowed the mass production of biologically active substances, and the development of rational drug delivery systems to control the disposition of these substances is needed. Liposomes are potential drug carriers, for these and other pharmaceuticals (Gregoriadis 1989; Juliano 1989; Scherphof et al 1989). The determinant factor in the disposition of liposomes is the uptake by the reticuloendothelial system, especially the liver. Quantitative analysis of this process is important in a rational integration of drug delivery systems.

This hepatic uptake process shows saturation (Abra & Hunt 1981; Kao & Juliano 1981; Bosworth & Hunt 1982; Beaumier & Hwang 1983; Chow et al 1989). This saturable process has usually been analysed by the Michaelis-Menten equation (Beaumier et al 1983; Sato et al 1986; Hwang & Beaumier 1988; Sculier et al 1989). The mechanism of liposome uptake is shown to be through endocytosis or phagocytosis (Finkelstein & Weissmann 1978; Straubinger et al 1983; Daleke et al 1990). Saturable uptake is one of the characteristics of endocytosis; however, it is questionable whether this process obeys Michaelis–Menten kinetics. Phagocytosis requires dynamic changes of plasma membrane, and the surface area of cells is kept constant by the recycling of plasma membrane (Steinman et al 1983). Therefore, saturation characteristics could be quite different from those of enzymatic reactions where the Michaelis-Menten equation is derived. In our previous paper, we focused on this point and observed a saturable process which was inconsistent with Michaelis-Menten kinetics by measuring the time courses of hepatic uptake of liposomes under different constant rates of infusion (Kume et al 1991). In our previous study, uptake clearance was calculated by assuming no efflux from tissue, and vascular space was not corrected for in the evaluation of tissue uptake of liposomes. In this study, the methodology of uptake clearance of liposomes was verified, and the kinetic analysis was performed on the determinant parameter which can explain saturable uptake clearance independent of infusion rate.

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Materials and Methods

Verification of the method for measuring uptake clearance of liposomes

Assumption of no efflux. If the efflux from tissue to blood can be neglected in the tissue distribution, the mass balance equation for the tissue is described as follows:

$$dX_t/dt = CL_t.C_t \quad (1)$$

where, X_t and C_t are tissue uptake and blood concentration at time t, respectively. Since the uptake clearance, CL_t, showed time dependency in the previous study (Kume et al 1991), the averaged uptake clearance from time 0 to t is defined as follows:

$$X_t = \int_0^t CL_t\, C_t dt \quad (2)$$

$$CL_t = \int_0^t CL_t\, C_t dt / \int_0^t C_t dt = X_t/AUC_{0\text{-}t} \quad (3)$$

where $AUC_{0\text{-}t}$ is $\int_0^t C_t dt$. In our preliminary study of bolus injection of liposomes, hepatic contents remained almost constant from 2 to 8 h after injection, while blood concentration was negligible after 30 min of injection. This result shows the absence of efflux between 2 and 8 h after injection; however, we could not exclude the possibility of efflux in the initial 2 h after injection. Therefore, we examined whether efflux can be negligible in this phase, using the following repeated dose study.

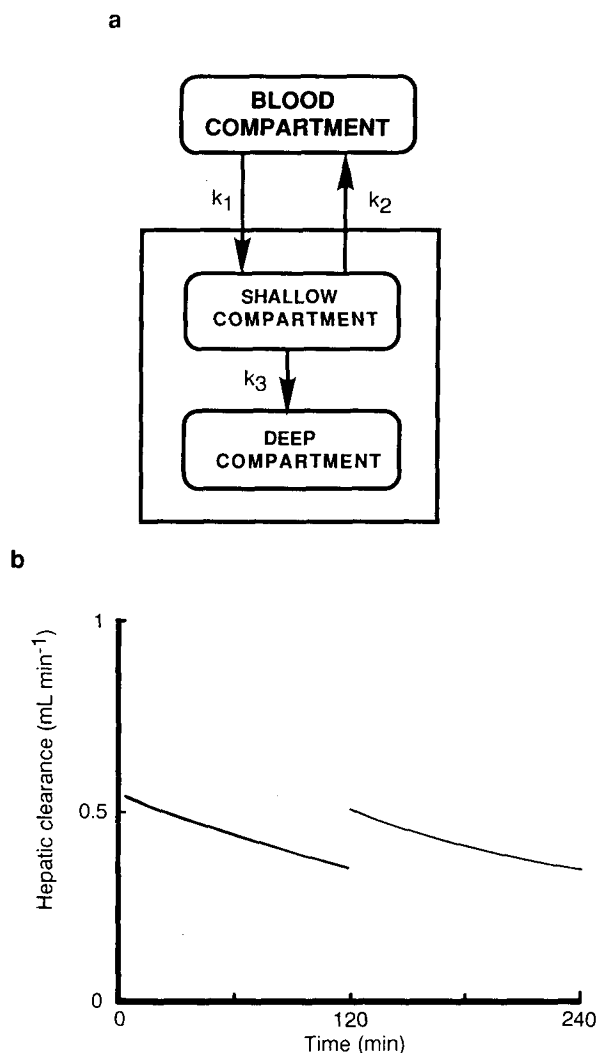


FIG. 1. a. Efflux model. Liver is divided into two types of kinetically different compartment; one is a shallow compartment with efflux to the blood compartment, the other is a deep compartment without efflux. Compartments are connected by the rate constants k_1, k_2 and k_3. b. Time course of hepatic clearance based on the efflux model. In a single dose study, labelled liposomes were injected intravenously. In a repeated dose study, unlabelled liposomes were first injected, then labelled liposomes were injected at 120 min. Hepatic uptake clearance was calculated with respect to the labelled liposomes, therefore the pattern is the same in both cases. Equations, initial conditions and values of parameters are described in the Appendix and Table 1.

Discrimination by repeated dose study: theory. Fig. 1a shows the hypothetical efflux model to be rejected in a repeated dose study. In this model, liver is classified into two kinetically different compartments; one is a shallow compartment with efflux and the other is a deep compartment without efflux. The uptake clearance was calculated using equation 3. The true uptake clearance is given as $k_1k_3/(k_2+k_3)$ for this model and this value is smaller than k_1, which we can obtain by neglecting efflux ($k_2=0$). According to this model, the uptake clearance calculated by equation 3 decreases with time due to the increase of efflux.

For clear understanding, simulation of uptake clearance was performed for a single bolus injection and a repeated injection. In a repeated-dose study, unlabelled liposomes were administered in the first dose then labelled liposomes were administered in the second dose. The uptake clearance based on equation 3 for both single and repeated administration was the same with respect to labelled liposomes, as the system is assumed to be linear and the effect of efflux occurs in the repeated administration in the same manner. Therefore, the uptake clearance increases discontinuously after repeated administration. If the decrease of uptake clearance was not due to the efflux but due to another mechanism such as saturation of clearance, then the discontinuous increase of uptake clearance should not be in evidence. The analytical solution for this model and the initial conditions for simulation are described in the Appendix.

Experimental procedure. The reagents used, the liposome preparation, surgery and assay were as described previously (Kume et al 1991). In brief, liposomes were prepared to give the lipid ratio of phosphatidylcholine (PC) /dicetyl phosphate (DCP)/cholesterol (CH) of 5/1/4 with a mean diameter of 300–350 nm. The trapping efficiency of inulin was 20–25%. Male Wistar rats, 250–300 g, were used. The operation for the introduction of catheters for drug administration and blood sampling were performed as described previously (Kume et al 1991).

Single dose study. Liposomes, labelled with [^{3}H]inulin (1500 nmol PC/300 g body weight) were injected intravenously. Approximately 300 μL of blood was sampled at the indicated times. At the end of the study (120 min), each rat was killed by the injection of 50 mg pentobarbitone and tissue was sampled.

Repeated dose study. Unlabelled liposomes (1500 nmol PC/300 g) were administered first, then the same dose of labelled liposomes was administered at 120 min. Blood and tissue were sampled after the second administration as described above. The radioactivities in blood and tissue were measured as described previously (Kume et al 1991). The uptake clearance was calculated according to equation 3.

Correction of vascular space. Vascular space was corrected in the evaluation of hepatic uptake.

Fig. 2 shows time courses of tissue to blood concentration ratio (K_p) for different infusion rates, using the data previously reported (Kume et al 1991). The higher the infusion rate, the lower the Kp. At the higher infusion rate, K_p ranged between 0·3 and 0·5, which is close to hepatic vascular space, 15% (Greenway & Stark 1971). In this analysis, each hepatic uptake was corrected by the vascular space as follows:

$$X_{cor} = X - C \cdot w \cdot 0 \cdot 15 \quad (4)$$

where, X and X_{cor} represent measured hepatic uptake and corrected hepatic uptake, respectively; w represents hepatic wet weight.

Kinetic characterization of the saturable uptake clearance Peviously reported data on the saturable clearance were used (Kume et al 1991). In brief, liposomes were infused at the rate of 4, 40 and 400 nmol PC min^{-1} to each rat for 6 h. Liposome

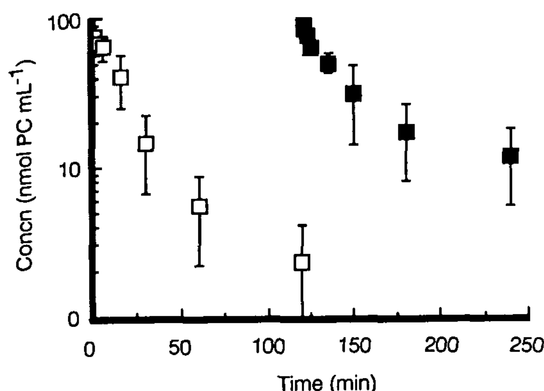


FIG. 2. Time courses of tissue to blood concentration ratio (Kp) in the liver. Each rat received constant rate intravenous infusion at the rate of 4 (●), 40 (■) and 400 (▲) nmol phosphatidylcholine (PC) min^{-1} and Kp-values were obtained at the indicated times (data from Kume et al (1991)). The vertical bar represents the s.d. (n = 3–5).

concentrations in blood and tissue were measured and uptake clearance was obtained using equations 3 and 4.

The determinant factors which explain the saturability of uptake clearance independent of the infusion rate were examined.

Mean blood concentration. The mean blood concentration was obtained as $AUC_{0\text{-}t}/t$. The relationship between mean blood concentration and hepatic clearance was examined to show whether hepatic clearance is determined by blood concentration as is the case with Michaelis–Menten kinetics.

AUC. First, the relationship between AUC and hepatic clearance was simulated. A one-compartment model was assumed with the infusion rate, k_o, Michaelis–Menten type elimination (K_m, V_{max}), and volume of distribution, Vd. Basic equations are as follows:

$$dC/dt = [k_0 - V_{max}\,C/(K_m + C)]/Vd \quad (5)$$

$$dX_t/dt = V_{max}\,C/(K_m + C) \quad (6)$$

$$AUC_t = \int_0^t C dt \quad (7)$$

$$CL = X_t/AUC_t \quad (8)$$

where V_{max} and K_m represent maximum uptake rate and Michaelis–Menten constant, respectively. The following values were employed for the simulation from the previous study: $V_{max} = 29{\cdot}7$ (nmol PC min^{-1}) and $K_m = 38{\cdot}8$ (PC mL^{-1}). A corresponding relationship was obtained from experimental data and compared with the simulated relationship.

Hepatic uptake amount. The relationship between hepatic uptake amount and hepatic clearance was examined to determine whether hepatic uptake can explain the saturation of hepatic clearance, independent of infusion rate. This examination was previously studied (Kume et al 1991) in the previous paper; however, the correction of vascular space had not been performed. Therefore this parameter was re-examined after the correction.

Numerical analysis. Curve fitting and simulation were performed on a personal computer using a nonlinear least square program, MULTI and the Runge-Kutta-Gill method (Yamaoka et al 1981; Yamaoka & Nakagawa 1983). The Damping Gauss Newton method was used as an algorithm for nonlinear least squares with inverse concentration weighting.

Results and Discussion

Repeated dose study

Blood. Time courses of blood concentration of liposomes in a single dose and a repeated dose study are shown in Fig. 3. Blood concentration decreased exponentially in both cases, however, these curves were not superimposable. The curve in a single dose study was well fitted by a one-compartment model and that in the repeated dose study was well fitted by a two-compartment model. Pharmacokinetic parameters are summarized in Table 1. A large difference (3·3-fold) was found in AUC between the single and the repeated dose study. As there is a big difference in the estimated AUC after 120 min between these studies, $AUC_{0\text{-}120}$ was also compared. As shown in Table 1, $AUC_{0\text{-}120}$ showed a 1·9-fold difference. There was little difference in the volume of distribution and $AUC_{0\text{-}60}$, but a 9-fold difference was observed in $AUC_{60\text{-}120}$. Therefore, this remarkable increase of $AUC_{60\text{-}120}$ indicates the saturation of uptake clearance during this period.

Hepatic uptake amounts. The hepatic uptake amount of liposomes increased with time as shown in Fig. 4. The lower uptake in the repeated dose study indicates the saturation of uptake clearance.

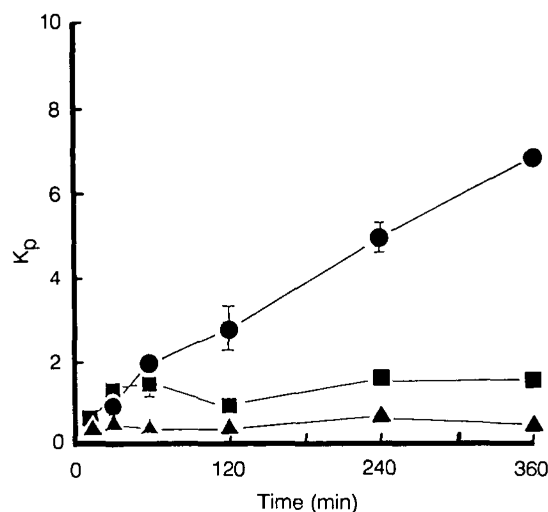


FIG. 3. Time courses of blood concentration in a single and a repeated dose study. In a single dose study (□), labelled liposomes (1500 nmol phosphatidylcholine (PC) 300 g) were intravenously injected. In a repeated dose study (■), unlabelled liposomes were first injected (1500 nmol PC 300 g), then labelled liposomes were injected at 120 min (1500 nmol PC 300 g). The concentration of labelled liposomes were measured. The vertical bar represents the s.d. (n = 3–5).

Table 1. Comparison of pharmacokinetic parameters between the single and repeated dose studies.

Parameters	Single dose	Repeated dose
Dose (nmol PC)	1500	1500[a]
A (nmol PC mL^{-1})	75.3 ± 4.4[b]	65.3 ± 12.2[b]
a (min^{-1})	0.0469 ± 0.0050[b]	0.0573 ± 0.0180[b]
B (nmol PC mL^{-1})	—[c]	22.0 ± 13.0[b]
b (min^{-1})	—[c]	0.0054 ± 0.0055[b]
AUC[d] (nmol PC min mL^{-1})	1605	5233
AUC_{0-120}[e] (nmol PC min mL^{-1})	1600	3083
AUC_{0-60}[f] (nmol PC min mL^{-1})	1509	2231
AUC_{60-120}[g] (nmol PC min mL^{-1})	91	851
CL[h] (mL min^{-1})	0.935	0.287
V_d[i] (mL)	19.9	17.2

[a]Unlabelled liposomes (1500 nmol phosphatidylcholine (PC)/300 g) were first administered, then labelled liposomes (1500 nmol PC/300 g) were administered. [b]Values represent mean ±s.d. (n = 3–5). [c]Blood concentration curve was analysed based on one-compartment model. [d]AUC represents the integral of C with time from 0 to infinity. [e]AUC_{0-120} represents the integral of C with time from 0 to 120 min. [f]AUC_{0-60} represents the integral of C with time from 0 to 60 min. [g]AUC_{60-120} represents the integral of C with time from 60 to 120 min. [h]CL represents total body clearance, and was calculated by dose/AUC. [i]Vd represents the volume of distribution for the central compartment and is calculated by dose/(A + B): for the single dose study, B = 0.

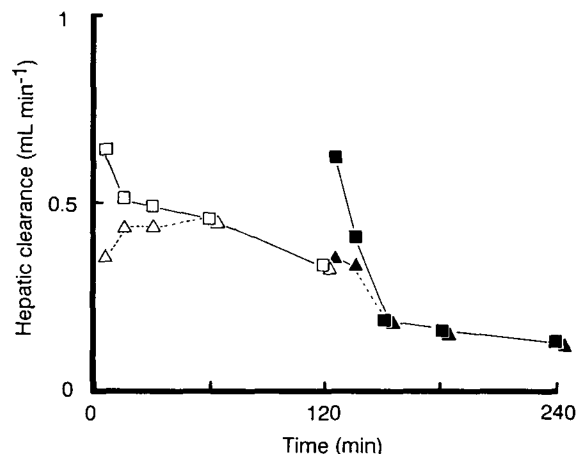


FIG. 5. Time courses of hepatic clearance in a single and a repeated dose study. Hepatic clearance was calculated by the equation 3. ■, □ represent uncorrected hepatic clearance, ▲, △ represent corrected hepatic clearance based on equation 4. □, △ represent the single dose study, ■, ▲ represent repeated dose study.

Hepatic clearance. The hepatic clearance is calculated according to equation 3 and both corrected and uncorrected hepatic clearance by the vascular space are shown in Fig. 5. The uncorrected hepatic clearance decreased with time in both cases and there was discontinuous increase at the second administration, while corrected hepatic clearance did not show the discontinuous increase. This result shows that the sign of efflux for the uncorrected hepatic clearance in the repeated study is an artifact due to the inclusion of liposomes in the vascular space into hepatic uptake. Therefore, there is little efflux with respect to liposomes, if any are actually taken up. The remarkable decrease of the uptake clearance in the repeated administration after 60 min corresponded well with the observed increase of AUC_{60-120}.

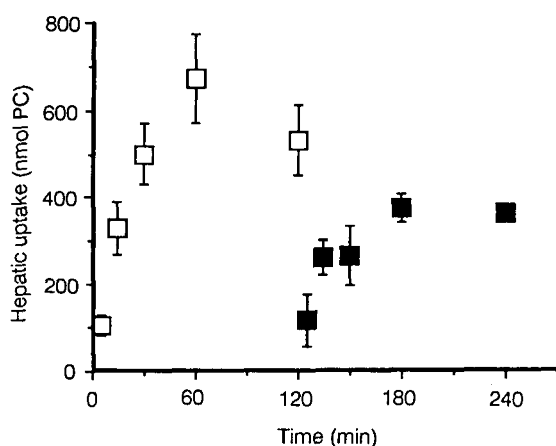


FIG. 4. Time courses of hepatic uptake of liposomes in a single dose (■) and a repeated dose (□) study. Experimental conditions are as described in Fig. 3. Whole uptake amount by the liver is shown as hepatic uptake. The vertical bar represents the s.e.m. (n = 3–5).

Kinetic characterization of saturable hepatic clearance

Mean blood concentration. The relationship between mean blood concentration and hepatic clearance is shown in Fig. 6. The hepatic clearance decreased with the increase of mean blood concentration for each infusion rate; however, each curve was separated by the infusion rate. This result is not consistent with Michaelis–Menten type kinetics, as uptake clearance should be described by the unique function of blood concentration.

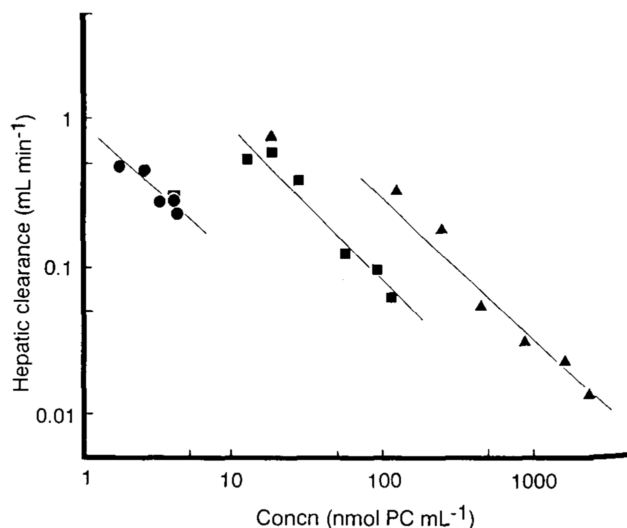


FIG. 6. Relationship between mean blood concentration and hepatic clearance. Experimental data of constant rate infusion study (Kume et al 1991) were used in this analysis. Mean blood concentration was calculated as described in Methods. ● 4, ■40 and ▲ 400 nmol phosphatidylcholine (PC) min^{-1}.

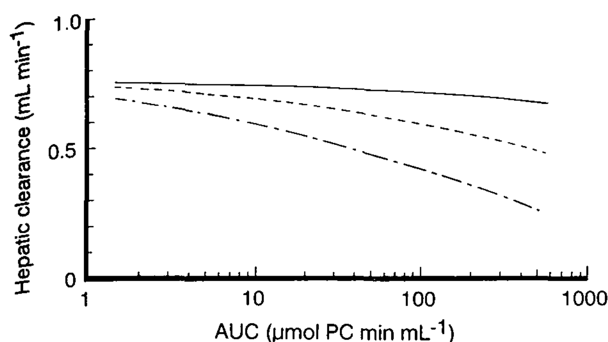
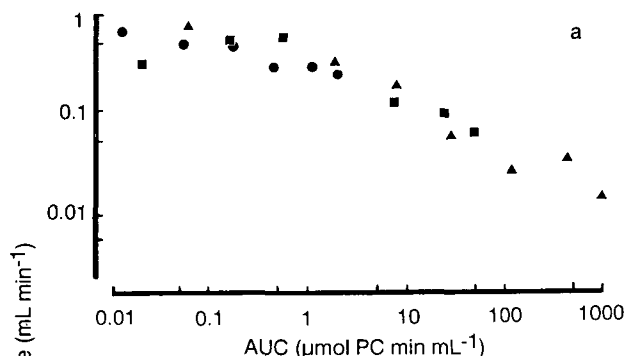


FIG. 7. Simulated relationship between AUC and hepatic clearance based on the Michaelis-Menten Model. Equations and conditions used in this simulation are described in Methods. Each line represents a different infusion rate. ——: 4 nmol phosphatidylcholine (PC) min^{-1}, - - - -: 40 nmol PC min^{-1}, -·-·-: 400 nmol PC min^{-1}.

AUC. The relationship between AUC and hepatic clearance was simulated based on the Michaelis-Menten model as described in Methods. As shown in Fig. 7, hepatic clearance did not decrease with AUC at the low infusion rate, however, there was a dependance of AUC on hepatic clearance at the higher infusion rate. Thus, the relationship between AUC and hepatic clearance strongly depended on the infusion rate. The corresponding relationship from the experimental data is shown in Fig. 8a. The hepatic clearance was uniquely described by the AUC within the experimental error. This shows that the saturability found in the hepatic uptake of liposomes is distinctly different from Michaelis-Menten kinetics. This result indicates the importance of exposure time as well as concentration in determining the saturability of hepatic clearance.



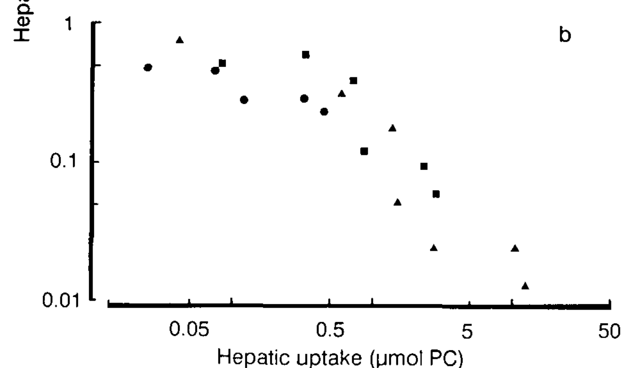


FIG. 8a. Relationship between AUC and hepatic clearance. Data are the same as those in Fig. 4b. The hepatic clearance was corrected by the vascular space of the liver according to equation 4. AUC was calculated by the trapezoidal method. Symbols are the same as those in Fig. 2. b. Relationship between hepatic uptake and hepatic clearance. Hepatic uptake was corrected for the vascular space according to equation 4.

Hepatic uptake. The relationship between hepatic uptake and hepatic clearance is shown in Fig. 8b. This relationship was independent of the infusion rate. In this study, both AUC and uptake amount could explain saturable uptake clearance of liposomes independent of the infusion rate. According to equation 3, there is a one-to-one relationship between AUC and uptake amount; we cannot distinguish which is the principal factor of this saturable uptake clearance without knowing the underlying mechanism.

Consideration of the underlying mechanisms. The mean diameter of liposomes used in this study was 300–350 nm; therefore, these liposomes may not pass the sinusoids to reach hepatocytes (Wisse 1970; Roerdink et al 1981; Poste et al 1982) and it is reasonable to assume that these liposomes were taken up through phagocytosis by Kupffer cells located around the sinusoids. Phagocytosis plays an important role in clearing large particles such as bacteria and aged red blood cells. Usually, complement receptors and Fc-receptors are involved in this process (Becker 1988; Juliano 1988). The process of phagocytosis consists of binding, engulfment, formation of phagosomes, fusion with lysosomes and digestion. This sequential process is similar to that of receptor mediated endocytosis; however, the size of particle taken up is different for phagocytosis and receptor mediated endocytosis, and the underlying mechanisms are also different (Darnell et al 1990). In addition, serum proteins called opsonins, which enhance the phagocytotic uptake, also play an important role in the phagocytosis (Saba 1970). The identification of opsonins and related receptors in the phagocytosis of these liposomes is now under examination in our laboratory.

The saturable clearance observed in this study can be qualitatively explained based on the internalization of receptors during phagocytosis as follows: assuming that the uptake clearance is proportional to the number of cell surface receptors for phagocytosis, and that internalized receptors are degraded and the synthesis of new receptors are slow compared with the uptake process, then the decrease of uptake clearance during phagocytosis is explained by the number of receptors expended.

On the other hand, the Michaelis–Menten equation can be derived for the initial rate of the reaction, assuming the steady-state condition for the enzyme-substrate complex. Therefore, it is reasonable that the Michaelis–Menten equation fails to explain the phagocytotic process where the total number of receptors on the cell surface is decreasing.

Further study is required on the mechanism of this uptake which is dependent on the saturable clearance of liposomes.

Appendix

The analytical solution for the concentration of each compartment for the efflux model was derived. The mass balance equation for a shallow compartment and a deep compartment are described as follows:

$$dX2/dt = k_1VdC - (k_2 + k_3)X2 \quad (A1)$$

$$dX3/dt = k_3X2 \quad (A2)$$

$$C = Ae^{-at} \quad (A3)$$

where X2 and X3 represent the amount in tissue per unit volume for a shallow compartment and a deep compartment, respectively, and C represents blood concentration. Equations A1–A3 were solved simultaneously, using Laplace transformation with the initial value of $X2(0) = X3(0) = 0$.

$$X2_t = P2e^{-at} + Q2e^{-(k_2+k_3)t} \quad (A4)$$

$$X3_t = P3e^{-at} + Q3e^{-(k_2+k_3)t} + R \quad (A5)$$

$$X_t = X2_t + X3_t \quad (A6)$$

where,

$$P2 = k_1Vd \cdot A/(k_2 + k_3 - a)$$

$$Q2 = k_1Vd \cdot A/(a - k_2 + k_3)$$

$$P3 = P2k_3/(-a)$$

$$Q3 = R2k_3/(-k_2 - k_3)$$

$$R = k_1k_3Vd \cdot A/(a(k_2 + k_3))$$

Kinetic parameters, k_1, k_2 and k_3 were obtained by the nonlinear least square method (MULTI), using the time course of hepatic uptake and blood concentration in a single dose study (see Figs 3, 4, and Table 1). Obtained values were as follows: $k_1 = 0{\cdot}0255$, $k_2 = 0{\cdot}00398$ and $k_3 = 0{\cdot}00147$.

Hepatic uptake was simulated using these parameters and other parameters in Table 1, hepatic clearance was also calculated according to equation 3.

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