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PHARMACOKINETICS AND TISSUE UPTAKE OF DOXORUBICIN ASSOCIATED WITH ERYTHROCYTE-MEMBRANE:ERYTHROCYTE-GHOSTS vs ERYTHROCYTE-VESICLES

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ABSTRACT

This study examined the usefuless of erythrocyte-membrane as a biodegradable carrier for intravenous injection of doxorubicin (DOX). Two different preparations of erythrocyte-membrane were used, erythrocyte-ghosts and erythrocyte-vesicles. Ghosts were prepared from red blood cells by hemolysis and repetitive washing until complete removal of hemoglobin. Erythrocyte-vesicles were prepared by ultrasonication of the ghosts suspension by a sonic dismembrator. The membrane products were then incubated with DOX before the injection. In CD rats, the disposition of DOX solution (DOX in normal saline) and erythrocyte-vesicles-DOX followed a two-compartment open model, whereas the ghosts-DOX exhibited a three-compartment characteristics. The area under the curve of the amount in the heart vs time for ghosts-DOX was approximately the same as for the solution. However, the amount of DOX in the heart after erythrocyte-vesicles-DOX injection was below the sensitivity of the

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detection. The fraction of DOX excreted unchanged in the urine was 0.54 for ghosts-DOX, 0.22 for DOX solution and 0.06 for erythrocyte-vesicles-DOX. The uptake of the drug by the spleen was increased after the administration of erythrocyte-vesicles-DOX. The observed and the calculated data address the usefulness of these delivery systems for DOX.

INTRODUCTION

Doxorubicin (DOX) is a potent anthracycline antibiotic anticancer drug which is effective against most hematological and many solid tumors. The most important side effect of the drug is cardiotoxicity. In man, this cardiotoxicity is manifested in the form of congestive heart failure (CHF). The CHF is especially seen when the cumulative dose of DOX exceeds 550 mg/m^2 (8). Various theories have been proposed to explain this side effect. Among them i) intercalation of DOX or its metabolites with DNA ii) lipid peroxidation of the plasma membrane, iii) increase in intracellular calcium concentration, and iv) effect on mitochondria are the most accepted.

The pharmacokinetics of DOX in animal models and in man have been described by a two-compartment open model (2,5,9). The drug distributes to various organs such as the heart, liver, lungs, spleen, and kidneys (1,3,4,11,13,14). The main metabolite of DOX is doxorubicinol (5). The other metabolites, DOX-aglycones, are found mainly in the liver at

approximately 3 hours post injection in Sprague-Dawley rats (15). The range or total urinary excretion of the unchanged drug is between 28.0 to 51.1% with normal renal function (12). The systemic clearance of DOX has been reported to be approximately 504 ml/min/m^2 (12).

To prevent the cardiotoxicity and/or to target the drug, various carriers have been proposed. Among them are microspheres, DNA, nanoparticles, and liposomes. The carriers can modify, to some extent, the disposition of the drug. For example, the initial plasma concentrations after DNA-DOX administration have been shown to be much higher than those of DOX solution and the uptake was mainly by the kidneys, lungs, duodenum, heart, stomach, and spleen. Or, in the case of liposomes, the major sites of the uptake were the tissues of the reticuloendothelial system.

The disposition of drugs associated with erythrocyte-ghosts would also be different from that of drug solution. The reason for this assumption is that erythrocyte-ghosts would be recognized by the body as damaged or dead erythrocytes and would rapidly be removed from the circulation by the reticuloendothelial system (7). The present study was designed to investigate the pharmacokinetics and tissue distribution of erythrocyte-membrane-DOX.

MATERIALS AND METHODS

I. Materials

Doxorubicin HCL was purchased from Sigma Chemical Company, St. Louis, MO. Human erythrocytes were obtained from the American Red Cross (Dedham, MA). Sep-Paks were purchased form Millipore (Milford, MA). All chemicals and solvents were of analytical grade, except those used in high pressure liquid chromatography (HPLC) analysis which were of HPLC grade (Fisher Scientific, Springfield, NJ).

II. Methods

1. Preparation of the Dosage Forms

a. Erythrocyte-Ghosts-DOX (EGD). Erythrocytes were washed with isotonic phosphate buffer, and then lysed and washed repeatedly with hypotonic phosphate buffer. The erythrocyte-ghosts suspension was collected when the supernatant became colorless. Five ml of this suspension was incubated with five ml of DOX solution (2 mg/ml) at 37° C for 24 hours. Following the incubation, the tubes were centrifuged for 20 minutes at 23,000 x g. The pellet was washed three times with normal saline. The average amount of DOX taken up by erythrocyte-ghosts was approximately 2mg/5 ml of suspension (2.005 ± 0.355 mg, n = 15). Before the injection, enough normal saline was added to form a less viscous suspension with a concentration of 1 mg/ml.

b. Erythrocyte-Vesicles-DOX (EVD). Five ml of the colorless ghosts suspension was sonicated for 3 min by using a sonic dismembrator at an energy level of 50 (Artex, Model 150). The disrupted erythrocyte-ghosts, under electron microscopy, exhibited a well defined vesicle-shaped structure with sizes much smaller than erythrocytes. The incubation of erythrocyte-vesicles with DOX was carried out under the same conditions as EGD and the final preparation was stored at 4° C. The average amount of DOX taken up by erythrocyte-vesicles was approximately 1 mg/1 ml of suspension (0.97± 0.0073 mg, n = 4).

2. Stability studies

a. EGD. The suspension was stored in the refrigerator (4°C) for a period of 14 days. The amount of free DOX was determined periodically.

b. EVD. The release of DOX at 4°C was monitored over a period of 19 days.

The decomposition of DOX solution was also studied for three weeks and the data of part a and b were corrected with respect to this control. The data are presented in the result section.

3. Animals and Treatments

Male CD rats (200-250 g) were housed in groups of 2 to 3 rats in plastic cages. Food (Purina rat chow) and water were provided ad libitum. They were kept in a

well ventilated room with a temperature around 20°C. They were divided into three groups. The control group (45 rats) received doxorubicin solution (DOX in normal saline) (9.50 mg/kg), a second group (41 rats) received EGD suspension (8.50 mg/kg), and a third group (40 rats) were injected with EVD suspension (5 mg/kg). All rats received the dose via a tail vein. The animals were decapitated at time intervals of 3, 10, 20, 40, 80, 150, and 300 minutes. Blood was collected in a beaker containing 1 ml of sodium citrate solution (5% w/v) as an anticoagulant. The blood was then centrifuged for 10 minutes at 2,000 x g to separate the plasma. Different organs such as heart, spleen and kidneys were quickly removed and stored at -10°C for future analysis. Urine was collected at different time interval during 24 hours for each dosage form (4-5 rats /group). Urine and plasma were also stored at -10°C.

4. Extraction of DOX from biolical Samples

a. Plasma and urine. Sep-Paks pre-treated with methanol and water were used for extraction. A volume of plasma or urine was passed through Sep-Pak using methanol and the methanol layer was evaporated under vacuum. The dry residue was reconstituted in 200 ul of methanol and analyzed by HPLC. The percent recovery of this method was between 91-96% with human or rat plasma.

b. Tissues. The intact organs were homogenized using a vertical hand homogenizer (Potter Elevjhem type). Each tissue homogenate was then ultrasonicated for 1 minute by the dismembrator at an energy level of 60. The homogenate was then extracted at pH 9.0 with a mixture of chloroform:methanol 3:1 (3 times extraction, each with 4 ml). The chloroform layer was evaporated to dryness by vacuum. The dry residue was reconstituted in methanol and analyzed by HPLC. The percent recovery of this method was between 87-93% with rat liver.

5. HPLC assay

The HPLC was carried out by a Millipore/Waters HPLC system (Milford, MA) using a stainless steel u-Bondapak C-18 column (3.9 x 300 mm). The mobile phase consisted of 30% acetonitrile and 70% aqueous ammonium formate buffer (pH = 4.0). All measurements were done at a wavelength of 254 nm (6).

6. Pharmacokinetic Analysis

Initial estimates of pharmacokinetic parameters were obtained by stripping the serum concentration-time data. These initial estimates were then used to generate a best fit of the data by using both two- and three-compartment open models by nonlinear iterative least squares regression with NONLIN (10). The difference between measured and computer-fitted

concentrations of doxorubicin, coefficient of determination and Akaike Information Criterion (AIC) (16) were used to determine the appropriate model. The parameters and constants were calculated with the use of the classical equations related to the two- and three-compartment open models with i.v. bolus input in the central compartment. The equations are presented in the result section.

RESULTS

Before the injection to the experimental animals, the stability of the dosage forms was determined. The kinetics of DOX release from EGD at 4°C was linear. The overall release rate was 1.6% per day. The EVD suspension stabilized at a plateau level of approximately 5% (Fig. 1).

The plasma concentration versus time curve of DOX following the intravenous injection of DOX solution is presented in Fig. 2. The biexponential equation describing the observed plasma data for the curve was calculated as follows:

$$C(t) = 1.75\, e^{-0.52t} + 0.034\, e^{-0.000897t} \quad (r^2 = 0.998)$$

Where C(t) is the dose-weight normalized plasma concentration at time t (min).

A similar mode of decline in the plasma concentration-time data was observed after the injection of EVD (Fig. 3). However, the concentration

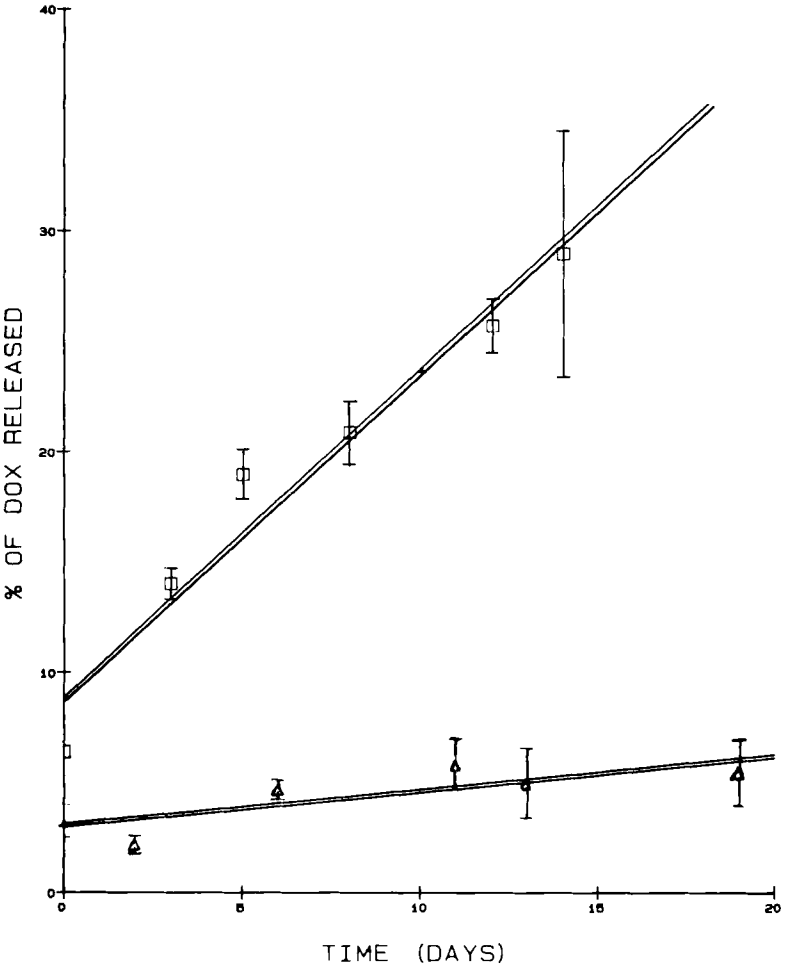

Fig 1: Cumulative percent of DOX released from EGD (□) or EVD (△) at 4°C over two and three weeks, respectively. The release of DOX from EGD was linear with T 1/2 = 12.5 days. the EVD was more stable with a plateau level of 5%.

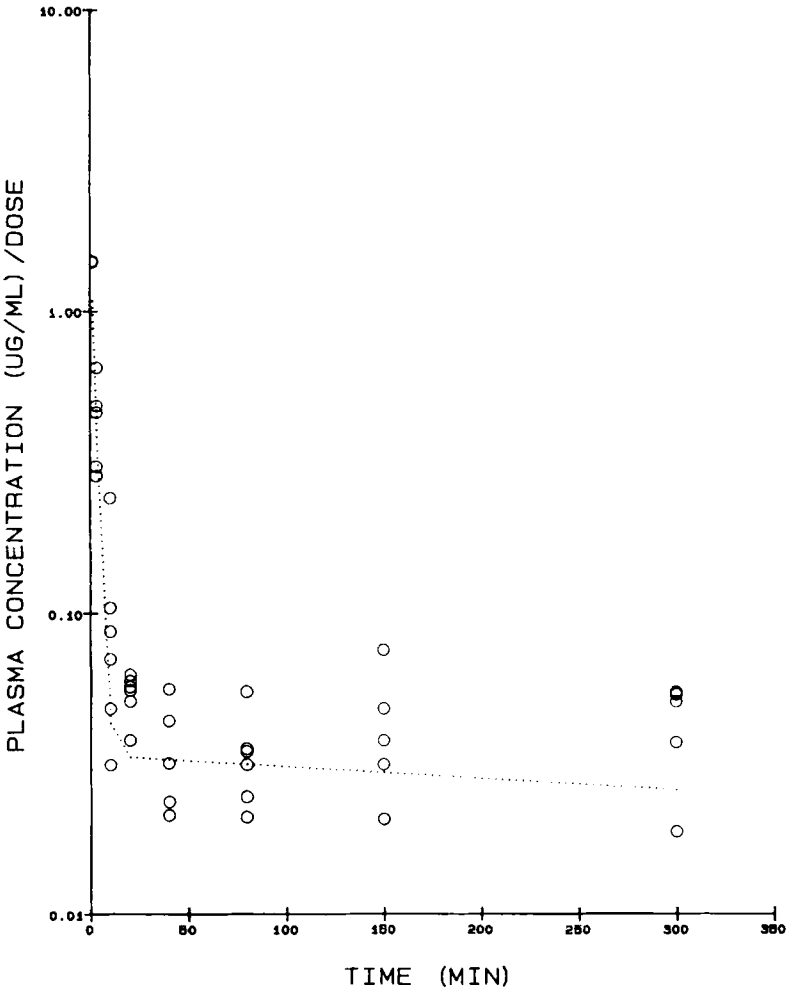

Fig 2: Normalized plasma concentration [(ug/ml)/dose (mg/kg)] vs time (min) after an i.v. injection of DOX solution (9.5mg/kg) to CD rats. The dotted line represents the calculated curve for the two-compartment open model.

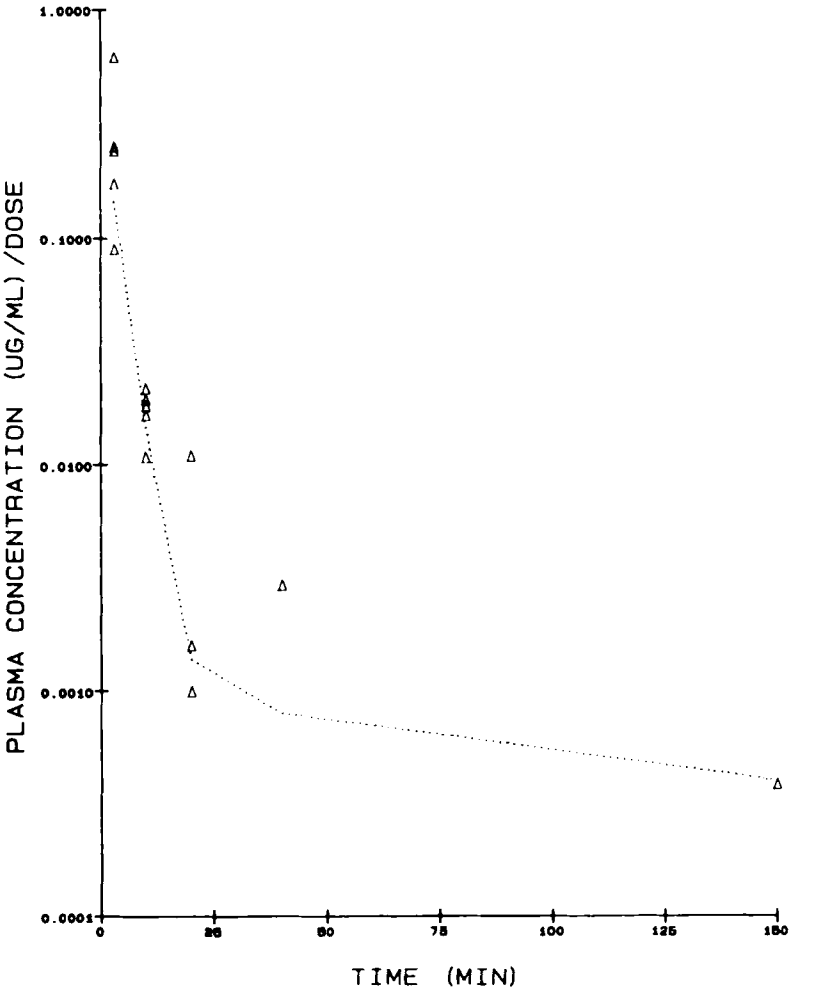

Fig. 3: Normalized plasma concentration [(ug/ml)/dose (mg/kg)] vs time (min) after an i.v. injection of EVD suspension (5mg/kg) to CD rats. The dotted line represents the calculated curve for the two-compartment open model.

of DOX at 40, 150 and 300 min for each rat was below the sensitivity of the HPLC detector. Therefore, it was decided to pool the six plasma samples together and then carry out the extraction. The equation representing the data for EVD was calculated as follows:

$$C(t) = 0.039\ e^{-0.336t} + 0.00102\ e^{-0.00627t} \quad (r^2 = 0.999)$$

Where C(t) is the dose-weight normalized plasma concentration at time t (min).

The plasma concentration-time data of EGD could not be fitted to a biexponetial equation, with or without weighting the data. The calculated line deviated significatly from the observed data points. However, based on the magnitude of the coefficient of determination and the Akaike Information Criterion (AIC) (16) the following triexponential equation was found to be sufficient to describe the data (Fig. 4):

$$C(t) = 2.03\ e^{-0.25t} + 0.0352\ e^{-0.016t} + 0.0616e^{-0.0002t}$$

$(r^2 = 0.99)$

The AIC values for the biexponential and triexponential equations were 40.21 and 21.63, respectively.

The analysis of urinary data showed more of the drug was excreted unchanged after the injection of EGD (Fig.5). The pharmacokinetic parameters and constants are presented in Table 1.

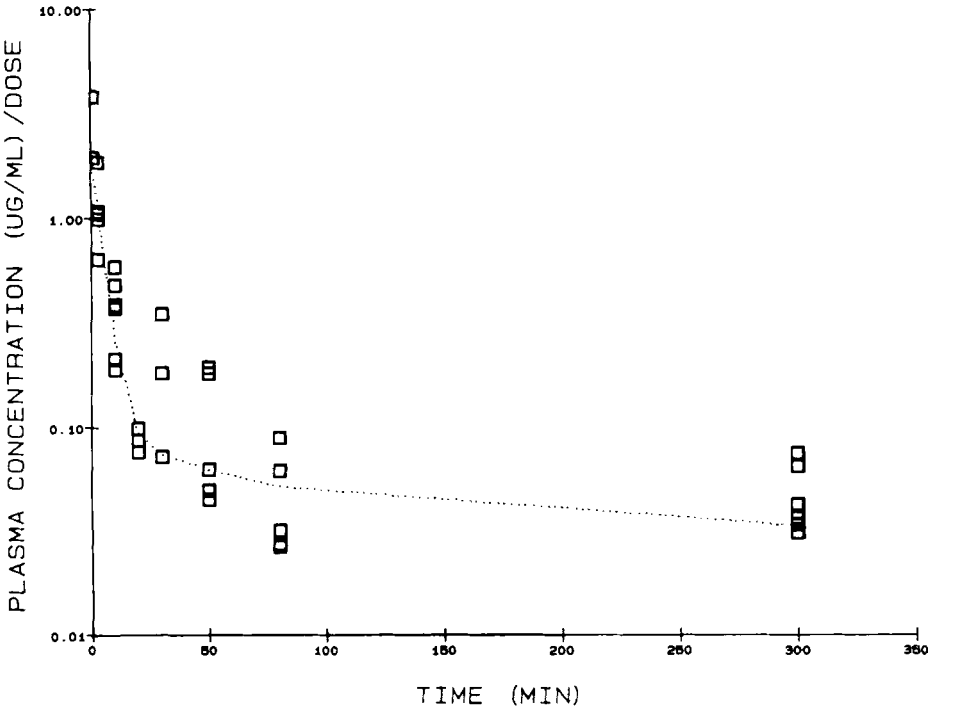

Fig. 4: Normalized plasma concentration [(ug/ml)/dose (mg/kg)] vs time (min) after i.v. injection of EGD suspension (8.5 mg/kg) into CD rats. The dotted line represents the triexponential fit for the data.

The time courses of DOX, EVD and EGD in different to organs were also investigated. The data are presented in Table 2. and Fig 6-9.

DISCUSSION

The results obtained in the present study provide strong evidence for the effect of erythrocyte membrane carrier on the disposition of doxorubicin. The data presented for EGD were based on a triphasic elimination

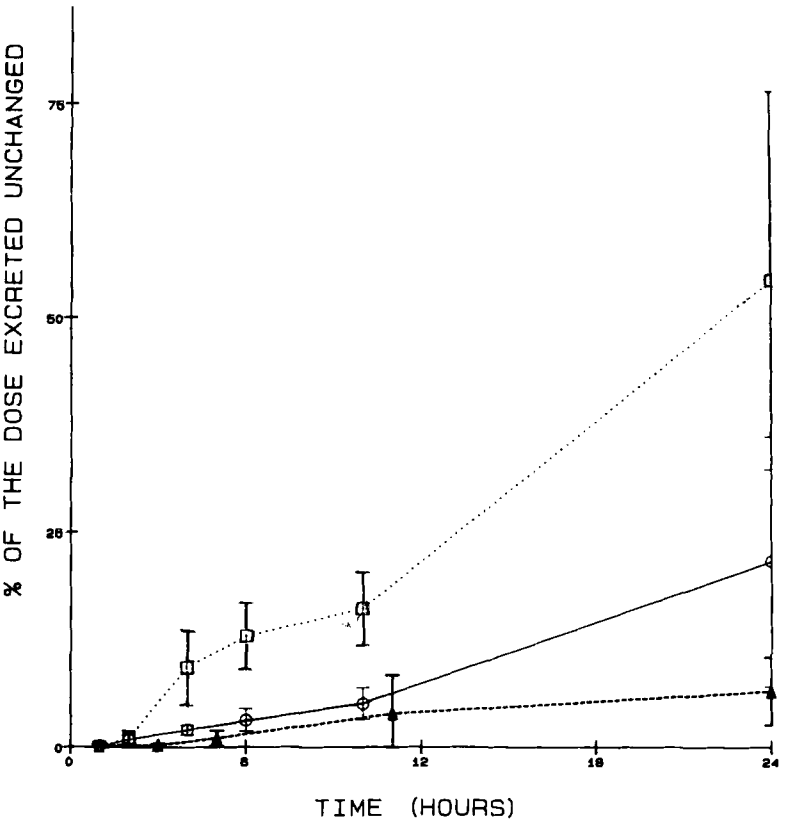

Fig. 5: Cumulative amount of unchanged DOX excreted in urine over 24 hours expressed as percent of the dose. EGD (...□...), DOX solution (——○——) and EVD (... △ ...).

pattern. However, for DOX solution and EVD the elimination kinetics fitted a biphasic model. The area under plasma concentration-time curve of the drug increased markedly (4.5-fold) following the administration of EGD. The total area (AUC_0^∞) under the plasma concentration-time curve seemed to be dependent upon the dosage form. The values of

Table 1. PHARMACOKINETIC PARAMETERS AND CONSTANTS OF DOX SOLUTION, ERYTHROCYTE-VESICLES-DOX (EVD) AND ERYTHROCYTE-GHOSTS-DOX (EGD) IN CD RATS.

	DOX SOLUTION	EGD	EVD
V_1 (l/kg)	0.550	0.470	2.550
V_β (l/kg)	27.050	15.750	120.470
V_{ss} (l/kg)	24.700	15.270	16.740
TBC (l/min.kg)	0.024	0.003	0.750
ke (min^{-1})	0.009	0.003	0.020
k_{12} (min^{-1})	0.467	0.079	0.040
k_{21} (min^{-1})	0.011	0.021	0.007
k_{10} (min^{-1})	0.043	0.006	0.295
k_{13} (min^{-1})	----	0.149	----
k_{31} (min^{-1})	----	0.005	----

TBC = Total Body Clearance
ke = Excretion Rate Constant
V_1 = Volume of the Central Compartment
V_β = Dose/λn AUC (λn = smallest hybrid rate constant, AUC = Area under plasma concentration-time curve)
k_{10} = Overall elimination rate constant.
k_{12}, k_{21} = Distributional rate constants between compartment 1 and 2
k_{13}, k_{31} = Distributional rate constants between compartment 1 and 3
V_{ss} = Apparent volume of distribution at steady state.

Table 2. AREA UNDER CONCENTRATION-TIME CURVE OF DOX EVD AND EGD IN DIFFERENT ORGANS OF CD RATS.

	DOX SOLUTION	EGD	EVD
Heart (ugh/g.dose)	1.43	1.35	*
Spleen (ugh/g.dose)	0.89	1.29	0.34
Liver (ugh/g.dose)	0.37	1.09	0.58
Kidneys (ugh/g.dose)	0.03	0.033	0.34

* not detectable

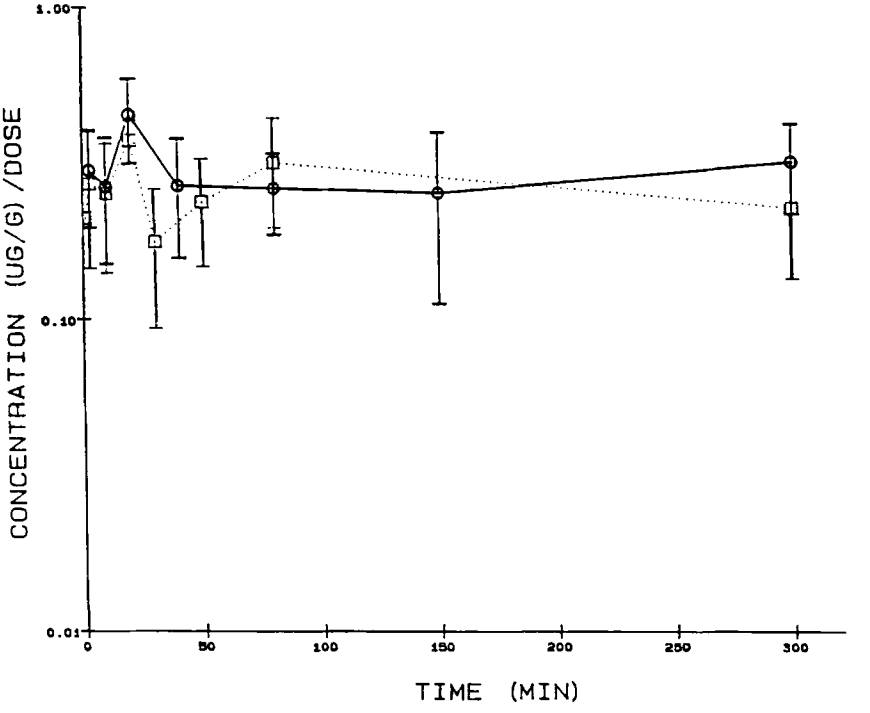

Fig. 6: The normalized DOX levels of the heart [(ug/g tissue)/dose (mg/kg)] vs time (min) after an i.v. injection of DOX solution (9.5 mg/kg)(—○—) or EGD (8.5 mg/kg) (...□...).

the distribution rate constants (k_{12}, k_{13} and k_{31}) also suggest that doxorubicin, given with the membrane carriers, remained longer in the central compartment. Based on the values of the distribution rate constants, the volumes of distribution and the overall elimination rate constant, it is obvious that the association of doxorubicin with erythrocyte membrane has modified the dispositional pattern of the drug.

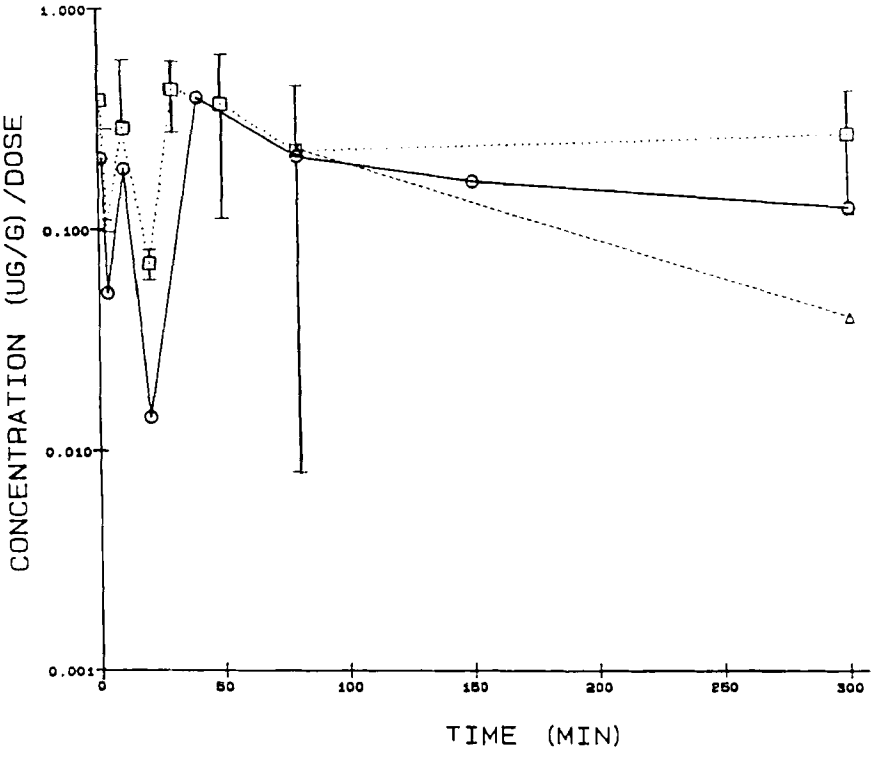

Fig. 7: The normalized DOX levels of the spleen [ug/g tissue)/dose (mg/kg)] vs time (min) after an i.v. injection of DOX solution (9.5 mg/kg) (—○—), or EGD (8.5 mg/kg) (...□...), or EVD (5 mg/kg) (...△...).

The fraction of doxorubicin excreted unchanged in the urine was 0.54 for EGD, 0.22 for DOX solution and 0.06 for EVD. However, the area under the concentration-time curve of the kidneys' uptake after the injection of EGD was comparable to the control. Whether the increase in the fraction of doxorubicin excreted unchange is the result of the uptake by the kidneys or a reduction in metabolism is not known. Doxorubicin and its metabolite doxorubicinol are

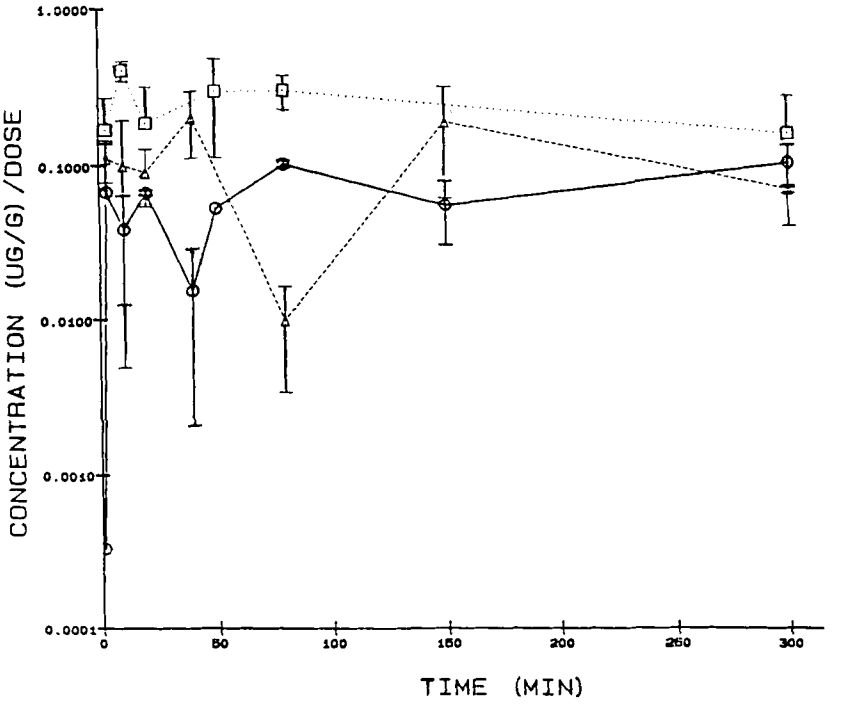

Fig. 8: The normalized DOX levels of liver [ug/g tissue)/dose (mg/kg)] vs time (min) after an i.v. injection of DOX solution (9.5 mg/kg) (—○—), or EGD (8.5 mg/kg) (...□...), or EVD (5 mg/kg) (...△...).

both metabolized, in part, by NADPH-dependent cytochrome P-450 reductase to yield the various aglycones. Higher fraction of unchanged drug in urine may be observed if cytochrome P-450 enzymes activity were decrease or the drug was protected from the enzyme system by the membrane. The significance of the higher fraction of dose excreted unchanged in the urine after the EGD injection is that the dosage form may be tailored for patients with urinary tract

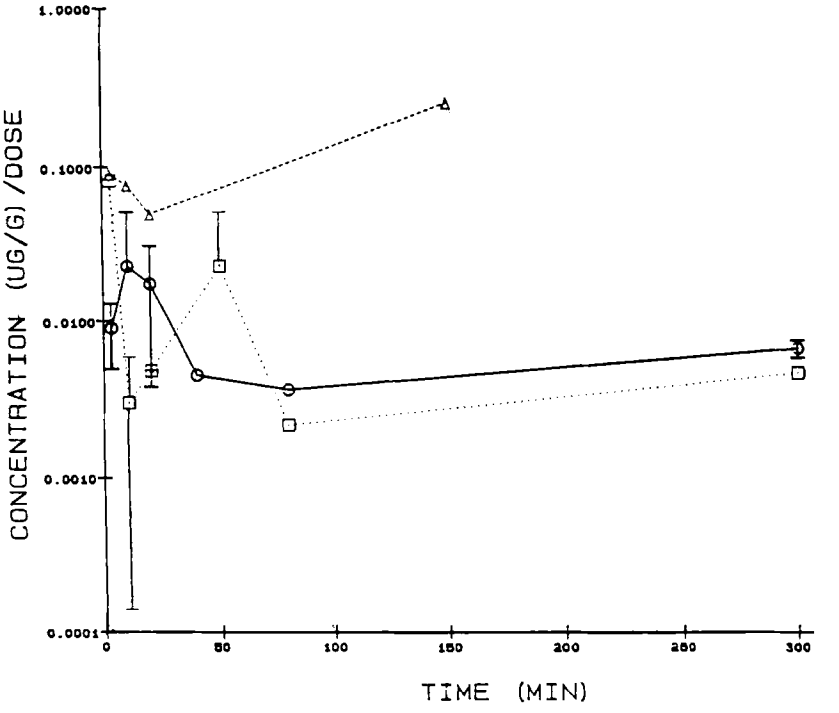

Fig. 9: The normalized DOX levels of kidneys [(ug/g tissue)/dose (mg/kg)] vs. time (min) after an i.v. injection of DOX solution (9.5 mg/kg) (—— ○ ——), or EGD (8.5 mg/kg) (...□ ...), or EVD (5 mg/kg) (...△ ...).

carcinoma to achieve potentially therapeutic but tolerable drug exposure.

The concentration of doxorubicin in each rat's heart after the injection of EVD was below the sensitivity of the detector. The initial thought was that this may be due to the lower dose of doxorubicin associated with EVD. However, after pooling the heart samples together (total of 6 per time point), extracting all doxorubicin, reducing the sample size by evaporation under nitrogen, the concentration

was still below the sensitivity of the detector. It was assumed that EVD may have reduced the uptake by the heart.

As it was expected, the concentration in the spleen was increased after the injection of EGD (Fig 8). However, the uptake of EVD by the spleen was less than control. It is evident that only intact erythrocyte ghosts are recognized by the body as damaged or dead erythrocytes.

The uptake of doxorubicin by the liver after the administration of EGD and EVD was higher than control. This may be due to the fact that particles of small sizes are preferentially taken up by the reticuloendothelial system.

In conclusion, the results indicate that the use of erythrocyte membrane as carrier may offer the advantage of localizing the drug in specific areas in the body such as liver, spleen and post-kidney urinary tract in the case of EGD, and avoiding areas, such as heart in the case of EVD.

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