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ADSORPTION ISOTHERM FOR DOXORUBICIN ON ERYTHROCYTE-MEMBRANE

Antoine Al-Achi and Mehdi Boroujerdi*

Section of Pharmaceutics
College of Pharmacy and Allied Health Professions
Northeastern University
Boston, MA 02115

ABSTRACT

The interaction of doxorubicin with erythrocyte-membrane was investigated to elucidate the mechanism(s) by which doxorubicin is taken up by erythrocytes. The uptake of intact and disrupted erythrocyte-ghosts was determined by using equilibrium dialysis. The adsorption isotherms revealed that the amount of doxorubicin adsorbed per unit weight of the erythrocyte-ghosts, at a given drug concentration, was similar to that of disrupted erythrocyte-ghosts. Also, the ratio of the calculated adsorption rate to desorption rate and the theoretical strength of the bonds between doxorubicin and the adsorption sites were the same for both systems. The results indicated that the uptake of doxorubicin by erythrocyte-ghosts may not be an encapsulation phenomenon, but rather an adsorption one.

INTRODUCTION

The potential usefulness of erythrocytes as a delivery system for therapeutic agents has been discussed

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*Author to whom corresponding should be addressed.

in a number of publications (1-9). The terms erythrocyte-ghosts are used in this study to describe the delicate discoid bodies obtained after the complete removal of the hemoglobin from the erythrocytes (10) (Fig. 1-a). The erythrocyte-ghosts membrane contains approximately 60% protein and 40% lipid by dry weight (11). About 30% of the lipids are cholesterol and the remaining 70% are phospholipds (12). The important protein of erythrocyte membrane is spectrin (13). The terms erythrocyte-vesicles will be referred to the small vesicles obtained after ultrasonication of erythrocyte-ghosts (Fig. 1-b).

The present study was designed to investigate the interaction of doxorubicin, an anthracycline anticancer drug (14-16), with erythrocyte-ghosts and erythrocyte-vesicles. Our objectives were to elucidate the nature of the uptake of doxorubicin by erythrocyte-ghosts and to determine whether the uptake is i) a passive diffusion (encapsulation) or ii) a physical adsorption.

MATERIALS AND METHODS

Materials

Doxorubicin HCl was purchased from Sigma Chemical Company (St. Louis, MO). Human erythrocytes were obtained from the American Red Cross (Dedham, MA). All other chemicals were reagant grade and those used for chromatography were HPLC grade. Dialysis membranes were

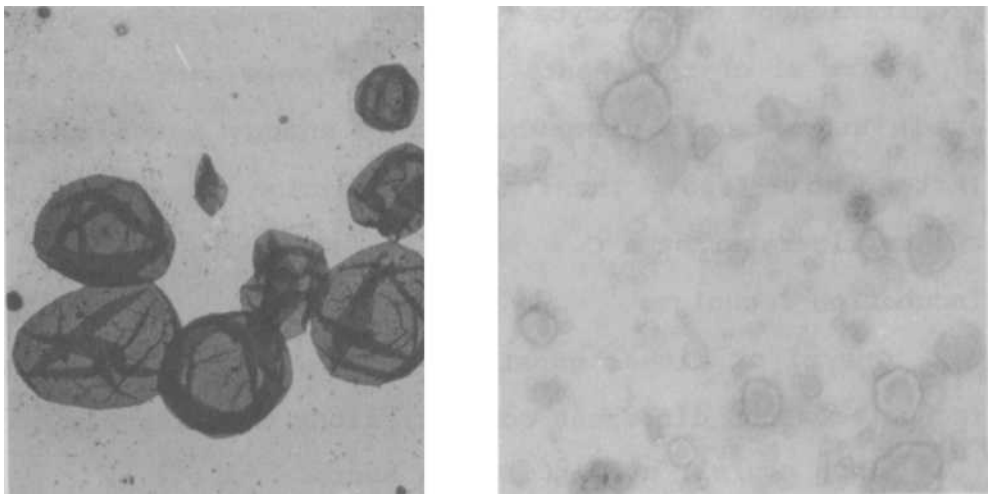

Fig. 1: Electron microscopy of a) Erthrocyte-ghosts (x4,000) b) Erythrocyte-vesicles (x46,000) using negative staining technique

purchased from Spectrum Medical Industries Inc. (Los Angeles, CA).

Preparation of Erythrocyte-Ghosts

One volume of human red blood cells was washed with an equal volume of isotonic phosphate buffer solution (pH = 7-7.2). The cells were lysed and washed several times with hypotonic phosphate buffer solution. After each wash, the solution was centrifuged for 20 min at 23,000 x g and the supernatant was aspirated and discarded. The ghosts suspension was finally obtained when the supernatant became colorless. The erythrocyte-ghosts were stored in the refrigerator at $4^{o}C$.

Preparation of Erythrocyte-Vesicles

Five ml of the ghosts suspension were sonicated for 3 min using sonic dismembrator at energy level of 50 (Artex, Model 150). The final preparation was stored in the refrigerator at 4°C.

Incubation Technique

One ml of either ghosts or eythrocyte-vesicles was incubated with different concentrations of doxorubicin (10 - 1500 mcg/ml) at 37°C for 24 hours.

Equilibrium Dialysis

The suspensions of ghosts or eythrocyte-vesicles, after the incubation with doxorubicin, were dialyzed (Spectra/Por #2 membrane, with 12000-14000 cut off) at 4°C for 24 hours against 200 ml of isotonic phosphate buffer solution. The concentration of free doxorubicin at 24 hours was determined and compared with a control (i.e., doxorubicin solution without ghosts or erythrocyte vesicles).

In another experiment, ghosts were incubated with doxorubicin. One half of the incubated suspension was ultrasonicated for 3 min using sonic dismembrator. Equilibrium dialysis was then carried out on both portions, and the amount of free doxorubicin was determined.

High Speed Centrifugation

Following the incubation of ghosts or erythrocyte-vesicles with doxorubicin, the suspensions were

centrifuged for 20 min at 23,000 x g. The amount of free doxorubicin in the supernatant was determined using HPLC. The results were then used to compare with the equilibrium dialysis data.

High Pressure Liquid Chromatography

The chromatographic analysis was carried out with Waters HPLC (Milford, MA) equipped with a stainless steel C-18 μ-Bondapak column (300 x 3.9 mm). Doxorubicin was analyzed isocratically using ammonium formate buffer: acetonitrile (70:30) as mobile phase with a flow rate of 2 ml/min for 15 minutes at 254 nm (17).

Determination of Number of Ghosts in the Suspension

The number of ghosts/ml were counted by using a Neubauer hemocytometer. Hypotonic phosphate buffer solution was used as the diluting solution.

Electron Microscopy

Ghosts or erythrocyte-vesicles were diluted with distilled water by mixing two parts of ghosts or erythrocyte-vesicles suspensions with 25 parts of distilled water. One drop of the diluted mixture was placed on a coated copper grid, then negatively stained with 1% phosphotungstic acid (pH=7.0). The material was tested on a Zeiss EM9S-2 electron microscope.

RESULTS AND DISCUSSION

The preparation of erythrocyte-ghosts suspension was reproducible and yielded, on average, four million

Table 1: Effect of temperature on the adsorption of doxorubicin on erythrocyte-ghosts

Temperature	Incubation Time (h)	% Bound (Mean ± S.D.)	n
25°C	24	33.79 ± 10.83	5
37°C	24	47.6 ± 8.52*	6

*Statistically significant difference between the two temperatures (P = 0.05)

ghosts/ml (n = 6, α = 0.05). The final wash had a colorless or a very faint pink supernatant which was indicative of complete removal of hemoglobin. No detectable damage to the ghosts was observed under electron microscopy (Fig. 1-a). When the erythrocyte-ghosts were ultrasonicated, the resulting preparation was easier to pour and less viscous. Electron microscopy revealed that the ultrasonicated solution contained vesicle shaped particles (erythrocyte-vesicles) (Fig. 1-b). The preliminary data of incubation on both preparations consistently showed a higher uptake of doxorubicin at 37°C for 24 hours than at room temperature for 24 hours (Table 1).

Based on the published data, at most 10% of the total amount of the drug bound to the membranes can be assumed to be due to the presence of spectrin (18). Furthermore, doxorubicin has been found to have a low

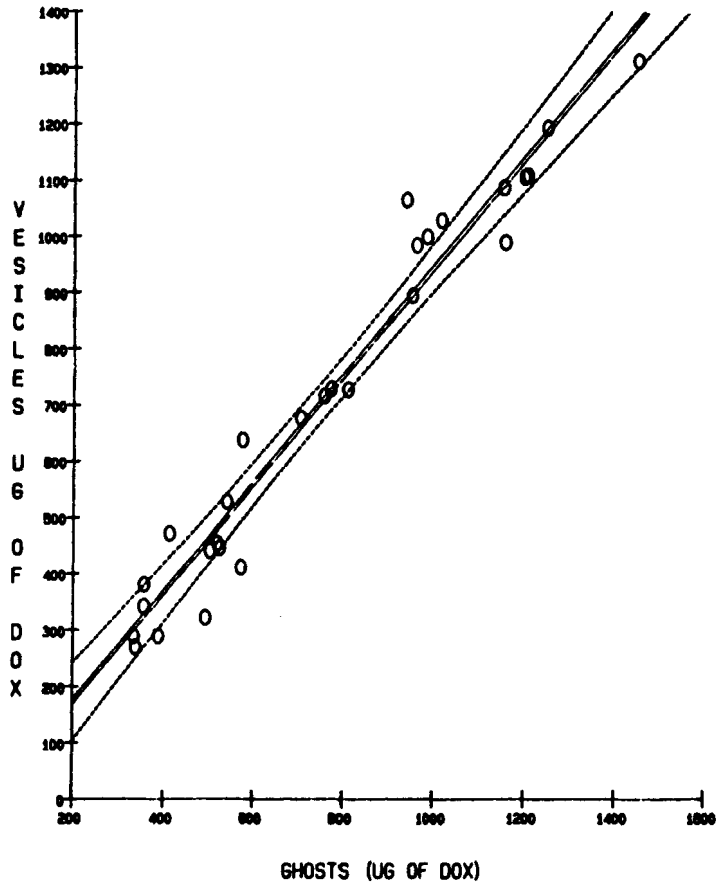

Fig. 2: The relationship between the amount of free doxorubicin in the dialysate after 24 hours. Erythrocyte-vesicles vs Erythrocyte-ghosts.

[y = 0.967x - 17.74 ; (r = 0.976)]

partition into the membrane phase of erythrocytes (19). Therefore, one may assume that the large uptake of doxorubicin by the erythrocyte-ghosts would be mainly encapsulation within the ghosts. To determine whether this assumption is correct, one half of a sample of

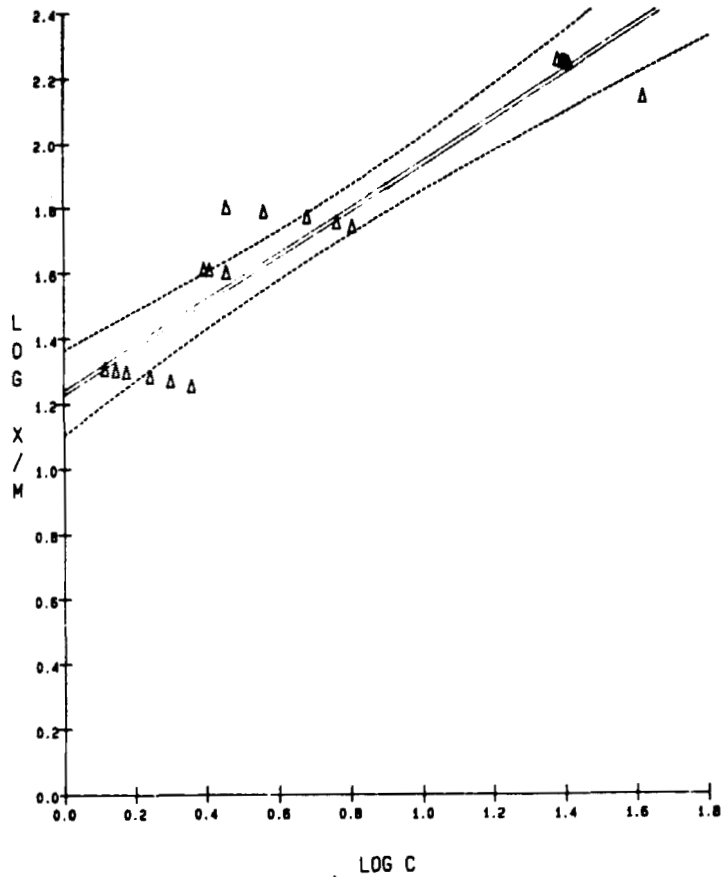

Fig. 3: Freundlich isotherms: The relationship between the log of amount of doxorubicin adsorbed per gram of a) ghosts and b) Erythrocyte-vesicles, versus the log of equilibrium concentration (mg%) using high speed centrifugation method.

Ghosts	log(X/m)=1.159 log C + 0.784	r=0.981
Erythrocyte-vesicles	log(X/m)=1.204 log C + 0.765	r=0.991

ghosts-DOX was ultrasonicated after the incubation and dialyzed as described in Materials and Methods. The equilibrium concentration of doxorubicin after dialysis was found to be the same as control (i.e., intact ghosts-DOX) (Fig. 2). This experiment indicated that there was

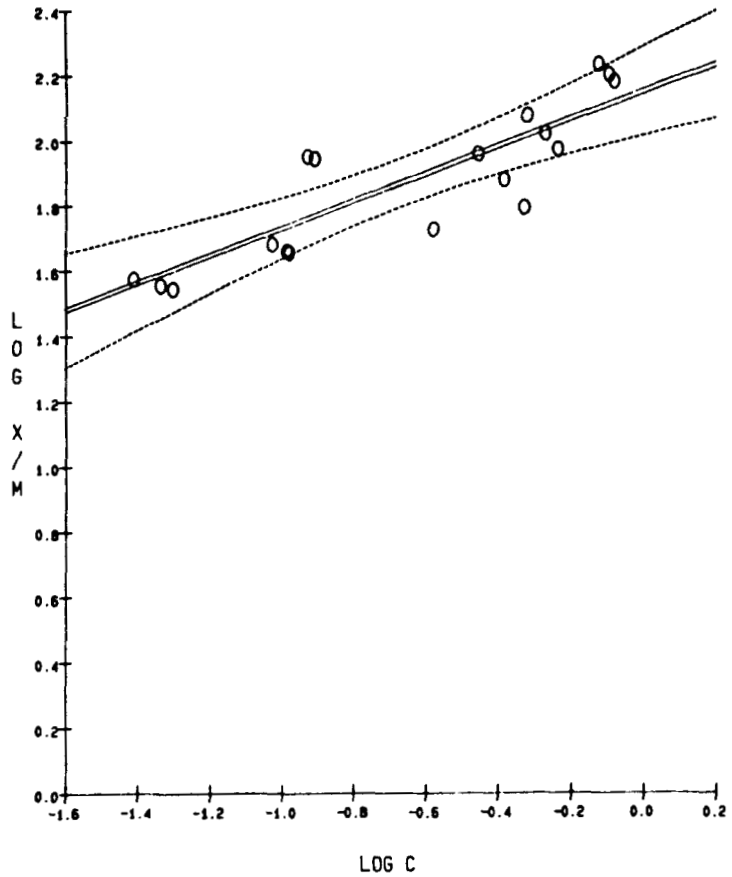

Fig. 3B

no or very negligible amount of free doxorubicin encapsulated inside the ghosts, otherwise the amount of free doxorubicin in the sonicated sample would have been higher than the intact ghost-DOX. Based on this observation, it was concluded that the uptake was mainly adsorption on the surface of the erythrocyte-ghosts rather than encapsulation.

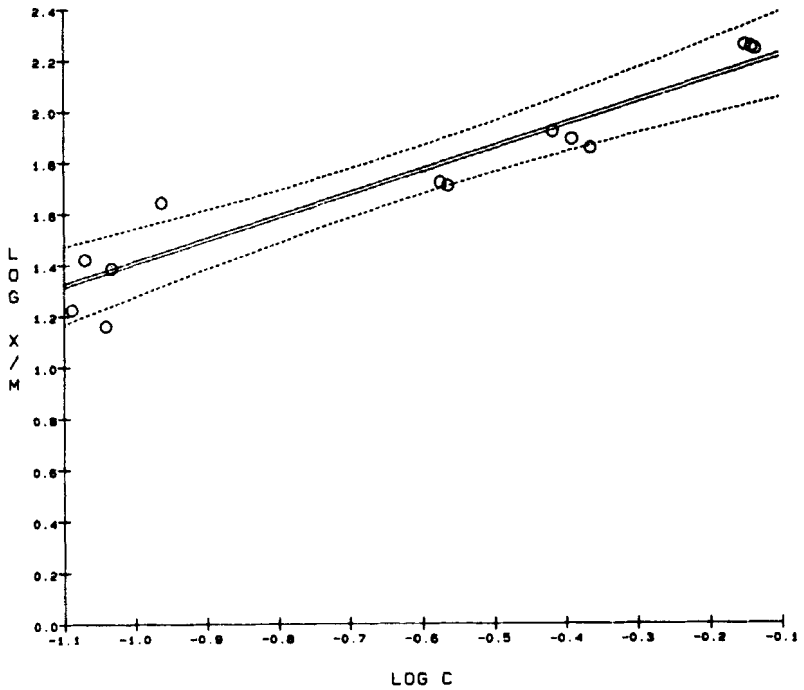

Fig. 4: Freundlich isotherms: The relationship between the log of amount of doxorubicin adsorbed per gram of a) ghosts and b) Erythrocyte-vesicles, versus the log of equilibrium concentration concentration (mg%) using equilbrium dialysis method.

Ghosts log(X/m)=1.051 log C + 2.466 r=0.931

Erythrocyte-vesicles log(X/m)=0.95 log C + 2.303 r=0.853

To study this adsorption phenomenon, the amount of doxorubicin bound to ghosts or erythrocyte-vesicles was determined by using both high speed centrifugation and equilibrium dialysis. The data were then fitted to Freundlich adsorption isotherm (Fig. 3 and 4) with the

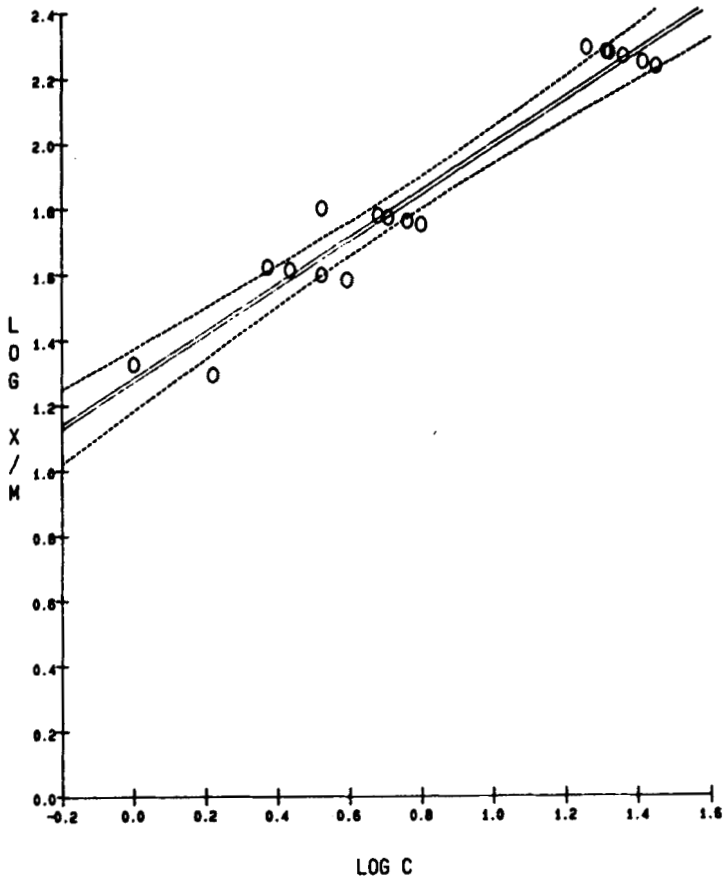

Fig. 4B

following general specifications:

$$\log(X/m) = \log k + n \log C$$

where X/m is the amount of doxorubicin adsorbed per unit mass of membrane (mg/g), C is the equilibrium concentration (mg%), k and n are constants.

The data in Table 2 indicate that the amount of drug adsorbed per unit weight of membrane at a given drug

Table 2: Comparison of Freundlich constants for adsorption of doxorubicin on erthrocyte-ghosts and erythrocyte-vesicles using high speed centrifugation and equilibrium dialysis

	High Speed Centrifugation		Equilibrium Dialysis	
Constant	Ghosts	E-vesicles	Ghosts	E-vesicles
n	1.16	1.2	1.05	0.95
k (mg/g)	6.080	5.820	292.41	247.17

Concentration range was 10-500 mcg/ml. The values were calculated by fitting the mean of measurements (6 samples/data point).

concentration, k, was similar for both systems, indicating that sonic disruption of the membranes did not alter the surface available for the adsorption. The large values of n would reflect a greater ratio of adsorbed molecules to free molecules of doxorubicin. It can also be related to the ratio of the rate of adsorption to desorption and the strength of the bonds between doxorubicin and the adsorption sites. The constant n for both systems (ghosts and erythrocyte-vesicles) was similar, indicating that ultrasonication did not change the nature of the adsorption sites.

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