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EFFECT OF COLLOIDAL CARRIERS ON THE DISPOSITION AND TISSUE UPTAKE OF DOXORUBICIN: I. CONJUGATION WITH OXIDIZED DEXTRAN PARTICLES

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ABSTRACT

Dextrans are water soluble polymers of glucose, with varying molecular weights. The free hydroxyl groups offer attractive sites for conjugation of drugs with the potential for altering the pharmacokinetic profile of the drug. Doxorubicin is a useful anticancer drug with cardiotoxicity as its most serious side effect. This drug was conjugated to dextran in the following manner. A Schiff's base was formed by incubating oxidized dextran (generated using sodium periodate) with doxorubicin. This mixture was then reduced using sodium borohydride. The conjugates, in the size range of 20 nm, were studied *in vitro* for the maximum uptake and the release of the drug. *In vivo*, the conjugates showed a markedly altered disposition profile from the control group. The total body clearance of the drug associated with the conjugate decreased. Additionally, lower concentrations of the drug were found in the heart of animals treated with the conjugates indicating the possibility of reduced cardiotoxicity.

INTRODUCTION

Doxorubicin is an effective anticancer drug that has been used in the treatment of solid tumors, leukemias, etc. Its clinical potential is limited by the development of

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cardiomyopathies. This cardiac toxicity is related to the cumulative dose administered. Its frequency markedly increases as the total dose exceeds 550mg/m^2 (1). We hypothesized that a controlled release parenteral dosage form of doxorubicin may reduce the frequency of cardiotoxicity even at the dose of 550mg/m^2. The water soluble dextran polymer was selected as the carrier due to its prolonged residence in the systemic circulation (2-9). The objectives of the study were: i) to investigate the influence of conjugation of doxorubicin with oxidized dextran on the plasma clearance and tissue uptake of the drug, ii) to determine whether the conjugation may decrease the uptake of the drug by the heart and potentially reduce the cardiotoxicity.

MATERIALS AND METHODS

CHEMICALS

Doxorubicin HCl, daunorubicin HCl, dextran of various molecular weights (10,000, 70,000, and 500,000), sodium periodate and sodium borohydride were obtained from Sigma Chemicals (St. Louis, MO). All other reagents and solvents were of analytical and HPLC grades. Dialysis Spectra/por cellulose membrane #3 with molecular cutoff of 3500, was purchased from Spectrum Medical Industries (Los Angeles, California).

CONJUGATION OF DOXORUBICIN TO OXIDIZED DEXTRAN

The method of preparation was optimized using dextrans of various molecular weights (MW 10,000, 70,000 and 500,000) and doxorubicin in varying amounts (0.2, 0.5, 0.8, 1.0 and 2.0 mg). Dextran was dissolved in 0.3M sodium periodate solution and stirred at 1000 rpm for one hour at room temperature. This represents a ratio of 1 mole periodate added per mole of glucosidic residues in the dextran and was considered a 100% oxidation. The oxidized dextran was dialyzed overnight in the cellulose bags against distilled water to wash out any excess oxidizing agent. Aldehydes generated by the oxidation of alcohol

groups on the dextran are reactive groups which react with the functional groups of the drug. Doxorubicin HCl, dissolved in 1 ml distilled water, was added to the oxidized dextran. The mixture was incubated in the refrigerator at 5°C for 5 hours. The Schiff bases thus formed, were reduced by addition of equimolar amounts of sodium borohydride. The reduction was carried out at 37°C for 2 hours. The treatment of the coupled material with sodium borohydride reduces any remaining aldehydes, blocking any further coupling. The conjugates of oxidized dextran and doxorubicin were examined under a transmission electron microscope to study the particle size of the conjugates. The electron microscopy revealed that the size of oxidized dextrans is independant of the molecular weight and is the same for the ones selected in this study (~ 20nm in diameter).

To determine the maximum uptake of doxorubicin by oxidized dextran, several solutions of increasing concentrations of doxorubicin with constant amount of oxidized dextran were used. The molecular weights of the dextrans studied were 10,000, 70,000 and 500,000 daltons. The control was a 10 ml mixture of doxorubicin and non-oxidized dextran. Doxorubicin ranging from 0.5 to 2.0 mg was added to 2 mg of oxidized or non-oxidized dextrans of all molecular weights. The dialysis bag was first washed in water for 4 hours to remove residual glycerin retained during manufacturing. Ten ml of the conjugated mixture or the control was placed in these bags. The bags were placed in plastic containers containing the dialyzing buffer. The control mixture of doxorubicin and non-oxidized dextran, was used to compensate for any osmotic pressure effects and Donnan equilibrium effects created by the presence of the dextran in the dosage form. Sealing the bags on both ends and covering the plastic container prevented loss of material due to evaporation. The dialysis buffer was 200 ml of pH 7.0 isotonic phosphate buffer.

The buffer outside the dialysis bag was sampled at 6, 12, 24, 30, 36,48 and 72 hours. The sample size was 1 ml which was replaced by fresh buffer. The contents inside

the bag were analyzed at the end of 72 hours. The analysis for the parent drug was done by chromatography. The amount of doxorubicin bound to the oxidized dextran was calculated as the difference in the amount of doxorubicin outside the dialysis bag between the control solution and the conjugated doxorubicin preparation. The amount of doxorubicin bound to the dialysis membrane was also determined and was between 5 to 10% of the amount added.

The release of doxorubicin conjugated with oxidized dextran was studied by using dynamic dialysis technique. The bag containing the dosage form was placed in 200 ml of isotonic phosphate buffer, pH 7.0, at room temperature. Samples were collected from the buffer at regular intervals over a period of 72 hours and analyzed for the drug.

IN VIVO EXPERIMENTS

Male CD rats weighing 175-200 g (Charles River Breeding Labs, Wilmington, MA) were allowed food and water *ad libitum*. After acclimation, the animals were randomly assigned to two groups, 40 animals in each group. The experimental design was parallel. The control group received doxorubicin in normal saline and the test group received doxorubicin conjugated with oxidized dextran. Both dosage forms were injected intravenously via the tail vein. The dosage forms were formulated to deliver 5 mg/kg of doxorubicin. The animals were sacrificed at 3,10,20,40,80,150 and 300 minutes and 24 hours after the administration of the dose. The blood samples were collected in plastic test tubes containing 0.5 ml saturated sodium citrate and centrifuged at 1000 g for 20 min to pelletize the cells. The supernatants were frozen and stored until assayed for doxorubicin by HPLC. The organs such as liver, lungs, spleen, kidneys and heart were removed immediately after the collection of blood samples and rinsed with distilled water, dried with paper towel and frozen.

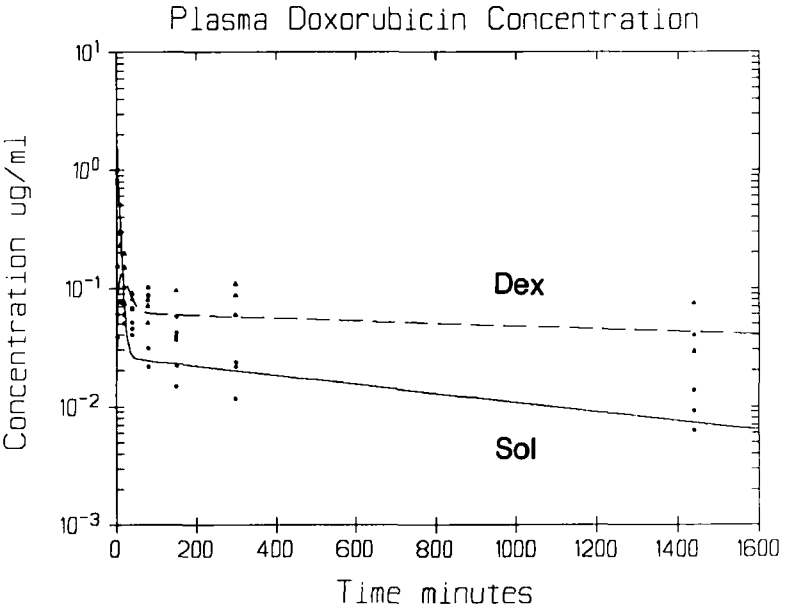

FIGURE 1.
Plasma concentration-time curve of doxorubicin administered in normal saline (_____) and associated with the oxidized dextran (--------).

The animals for the 24 nour time point were placed individually in metabolism cages with food and water provided *ad libitum*. The urine samples were then collected at 1, 2, 4, 6, 10, 12 and 24 hours after the injection of the drug and frozen. At each sampling time the bottom tray of the cage was rinsed with distilled water and added to the sample. After the last urine sample, the animals were sacrificed and the 24 hour blood and tissue samples were collected.

ANALYTICAL METHOD

The extraction of doxorubicin from biological samples was carried out with 4:1 chloroform methanol mixture. The tissue samples were homogenized before the extraction, using hand held homogenizer (Biospec products, Bartesville, OK). The organic phase from

the extraction was evaporated in silanized glassware to dryness under vacuum. The residue was reconstituted in methanol and analyzed by HPLC.

Doxorubicin concentrations were determined by HPLC (Waters, Milford, MA). The stationary phase was octodecylsilane (ODS). The mobile phase was a 70:30 mixture of methanol and ammonium formate, pH 4.0. The chromatography was isocratic with the flow rate maintained at 2 ml/min. The detection was carried out by fluorometry (Gilson 121, Gilson Instruments, Randolph, MA) with 470 nm excitation and 540 nm emission wavelengths. Daunorubicin, a closely related analog of doxorubicin, was used as the internal standard.

RESULTS

IN VITRO DATA

The amount of doxorubicin bound to the oxidized dextrans was determined and plotted against the initial amount of the drug which was added at the time of conjugation (Fig 2). Dextran 70 and 500 had greater uptake than dextran 10. The curves for dextran 70 and 500 showed similar pattern of uptake.

Plot of logarithm of amount of doxorubicin bound per unit mass of dextran (x/m) against the logarithm of concentration of doxorubicin in the solution at equilibrium shows a linear relationship as predicted by the Freundlich isotherm (Fig 3). The isotherms for all three molecular weight dextrans showed a good correlation ($r^2 = 0.99$) and similar slopes (0.71 to 0.74) (Table I). As the slope of the Freundlich equation expresses the affinity between the adsorbate and the adsorbent, the similar slopes may indicate similar affinity for the various oxidized dextrans by doxorubicin. The y-intercept of the equation represents the number of binding sites, and for dextran with molecular weight 10,000 the

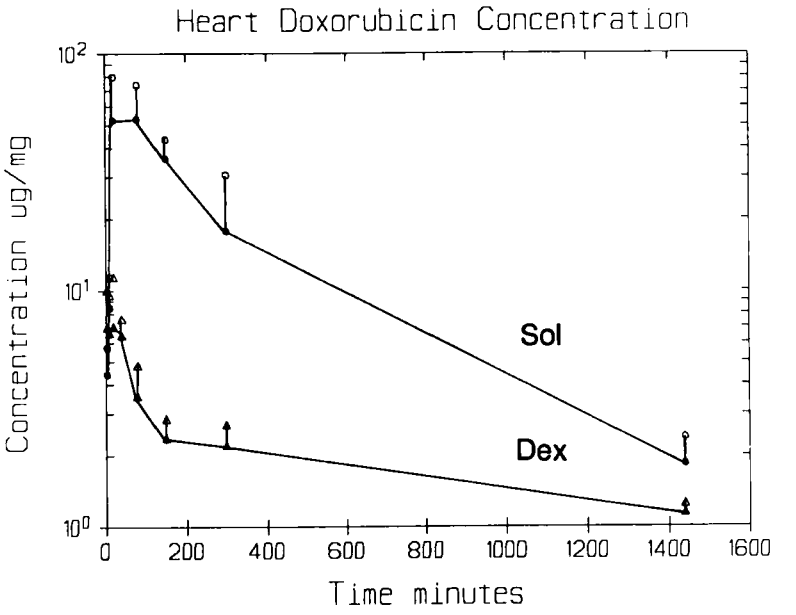

FIGURE 2.
Concentration-time profile of doxorubicin in the heart after the intravenous administration of doxorubicin in normal saline (o) and the oxidized dextran (●).

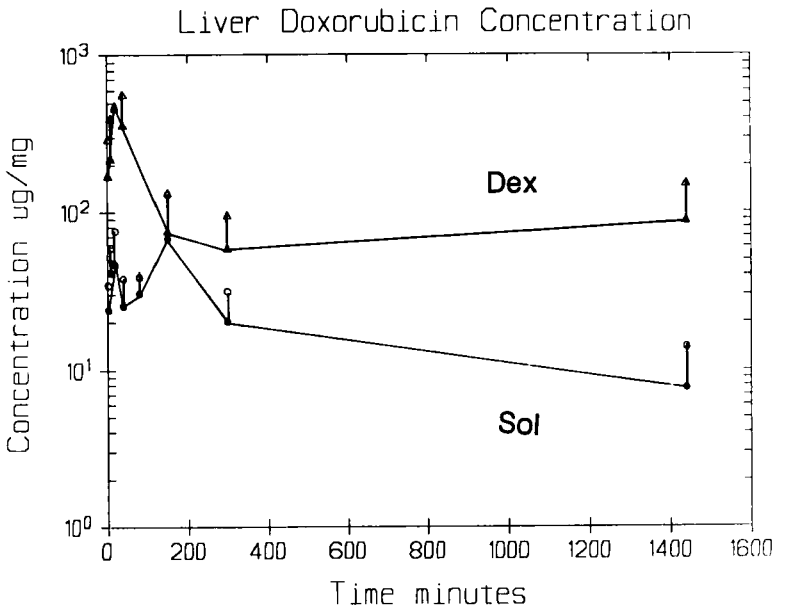

FIGURE 3.
Concentration-time profile of doxorubicin in the liver after the intravenous administration of doxorubicin in normal saline (o) and the oxidized dextran (●).

TABLE I

Uptake Data from the Linear Plots of Freundlich Isotherms

•Dextran Molecular Weight (thousands)	10	70	500
•Adsorption Isotherm plateau (μg/mg)	55	65	65
•Freundlich Isotherm			
- Slope	0.74	0.71	0.71
- Adsorptive capacity (y-Intercept)	1.65	5.24	5.24

intercept is 1.65 and suggests lesser binding sites than those on dextrans with molecular weights 70,000 and 500,000 whose intercepts are around 5.24 (Table I).

The data did not fit the Langmuir equation. As Langmuir isotherm is based on a uniform monolayer adsorption, the lack of fit may suggest that doxorubicin is adsorbed on heterogeneous sites on the oxidized dextran particles.

The binding capacity of dextran 70 was greater than dextran 10 and equal to dextran 500. Therefore, it was decided to use dextran 70 as the polymer for the dosage form and animal experiments.

The release of doxorubicin conjugated with oxidized dextran was studied in buffer solution using dynamic dialysis. The corresponding rate constants are:

$K_{conj} = 0.0124 hr^{-1}$ and $K_{free} = 0.0196 hr^{-1}$

The difference in the half-lives is approximately 20 hours. This indicates that

under the experimental conditions used in this study the release of the drug conjugated to oxidized dextran occurs at a much slower rate than the solution.

IN VIVO DATA

All plasma data were normalized with respect to the weight of the animal and the administered dose of the drug. The initial estimates of pharmacokintetic paramteres were obtained by striping the serum concentration-time data. These initial estimates were then used to generate a best fit of the data by nonlinear iterative least squares regression with PCNONLIN. The area under the plasma concentration-time curve (AUC) was calculated by the linear trapezoidal rule. The apparent volume of distribution (Vd) and total body clearance (TBC) were calculated as:

$$Vd = Dose / (AUC * \beta), \quad TBC = Dose/ AUC$$

where β is the terminal rate constant.

The calculated parameters and constants are reported in Table II. The plasma concentrations of the free drug after the administration of the oxidized dextran showed an initial rapid rise before a biphasic decline and were fitted to a two-compartment model with first-order release in the central compartment (Fig. 1).

The selection of the model was based on the Akaike value and the coefficient of determination. From a comparison of the plasma concentration-time curves, it is apparent that the drug associated with dextran had significantly longer circulation time than the drug in normal saline. The lower value of total body clearance and the higher AUC are due to the retention of the collidal dosage form in systemic circulation . The Mean Residence Time (MRT), calculated by Noncompartmental Analysis, supports the prolonged residence of the drug in the body when it is conjugated with oxdized dextran. The overall elimination rate constant k_{10} of the control group is in agreement with reported values (10).

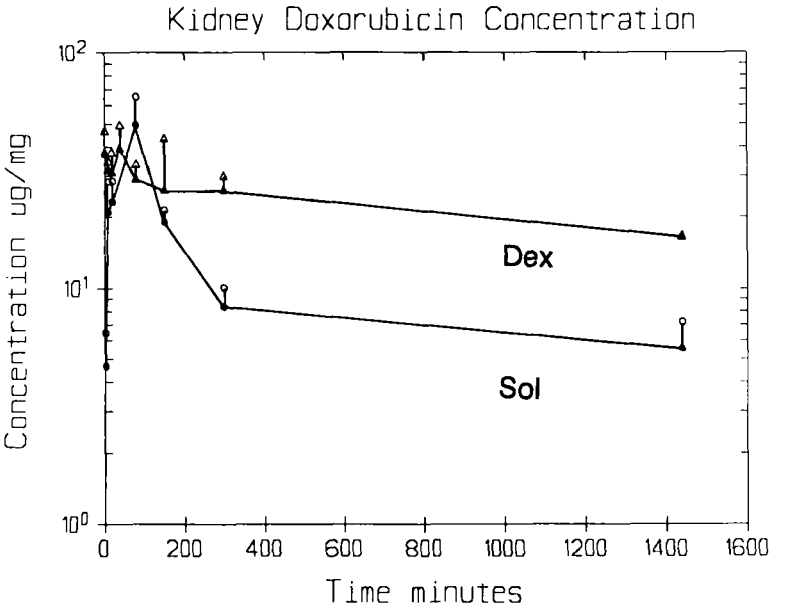

FIGURE 4.
Concentration-time profile of doxorubicin in the kidney after the intravenous administration of doxorubicin in normal saline (o) and the oxidized dextran (●).

The 24 hr excretion of doxorubicin in the urine of rats treated with dextran dosage form was approximately 50% of the control. The reduction is consistent with the higher plasma concentration and the lower total body clearance. The overall elimination rate constant, k_{10}, was also calculated from the urinary data by using the Rate plot. The calculated value, 0.058 h^{-1}, was similar to the calculated value from plasma data.

The time course of drug retention in heart, liver, spleen, lungs and kidneys are presented in Figs. 2-6. In Table III, the extent of tissue retention of free doxorubicin after the administration of each dose is expressed as area under the tissue concentration-time curve. As the excess blood was rinsed and wiped from the tissues, it was assumed that the data were corrected for tissue blood content. Peak concentrations in the liver were

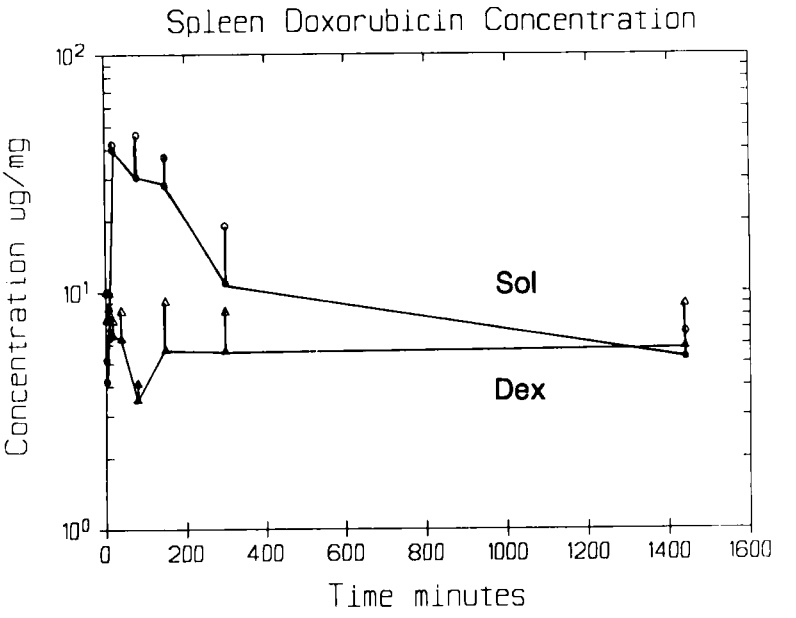

FIGURE 5.
Concentration-time profile of doxorubicin in the spleen after the intravenous administration of doxorubicin in normal saline (o) and the oxidized dextran (●).

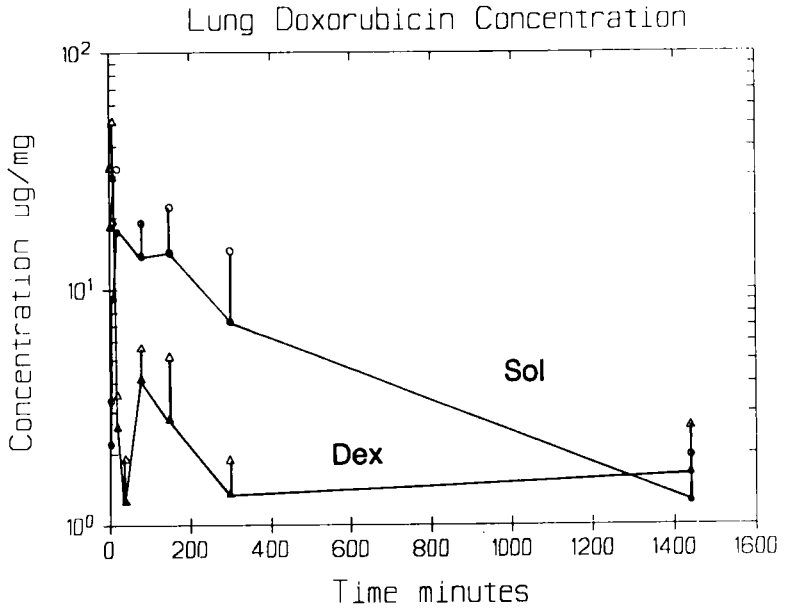

FIGURE 6.
Concentration-time profile of doxorubicin in the lung after the intravenous administration of doxorubicin in normal saline (o) and the oxidized dextran (●).

TABLE II

Comparison of Pharmacokinetic Parameters of Doxorubicin After Intravenous Injection of Free Doxorubicin (Control) and Doxorubicin Associated with Oxidized Dextran in Rats

	Control	Oxidized Dextran
$AUC_{(0-t)}$ (mg.min/l)	38.23	243.05
MRT (hr)	5.81	9.65
k_{10} (hr^{-1})	2.42	0.07
Vd (l)	29.74	16.03
Clearance (ml/min)	26.18	4.11

AUC: Area under plasma concentration-time curve from 0-t
MRT: Mean Residence Time
k_{10}: Overall elimination rate constant
Vd : Apparent volume of distribution

observed 20 minutes post injection and after 10 minutes in the kidney, spleen and lungs. In comparison to the control, higher levels of drug were found in the liver and kidney and lower levels were found in spleen and lungs. The terminal plateau levels of the liver were consistent with the profile of the plasma concentrations.

DISCUSSION

The concentration of doxorubicin in plasma after the adminstration of the dextran dosage form depends on the rate of breakdown of the conjugate, the rate of uptake by tissues and the rate of excretion of the drug. The drug is bound to oxidized dextran by chemical adsorption and the release of the drug *in vivo* from the carrier would be affected by a number of biological factors that can not be reproduced *in vitro*. The *in vivo* release

TABLE III

Comparison of the Area under the Tissue Concentration-Time Curves for Major Organs after the Intravenous Injection of Free Doxorubicin (Control) and Doxorubicin Associated with Oxidized Dextran in Rats

	Control (ug min/g of tissue)	Oxidized Dextran (ug min/g of tissue)
Heart	20.43 ± 11.78	2.90 ± 0.65 *
Liver	28.63 ± 19.41	119.49 ± 73.52
Kidney	24.58 ± 12.78	37.87 ± 5.21
Spleen	15.70 ± 8.34	8.15 ± 3.93
Lung	8.14 ± 6.84	2.76 ± 1.48

* $p < 0.05$

as determined by the concentration of the drug in the plasma shows a biphasic pattern. A burst effect is seen at the early time points followed by a subsequent slower release. The secondary slow release rate correlates better with the *in vitro* data.

There is an initial rapid drop in the level of cirulating doxorubicin due, in large part, to the rapid and significant initial uptake of oxidized dextran by the liver (and kidney to some extent). The liver has a great capacity for uptake of conjugates than does the spleen. In general, the conjugates are more selective toward the liver than other reticuloendothelial organs. The uptake by the heart and lungs is negligible. This may be due to the small size of the conjugates. The subsequent slower decrease in blood levels is related to slow elimination and tissue uptake of circulating conjugates which is controlled by the gradual breakdown of the conjugates.

The conjugates are retained longer in the systemic circulation and liver. It is apparent that the disposition rate constant of the drug associated with the dextran is a

function of the *in vivo* release rate and is a hybrid rate constant encompassing the release and disposition rates.

In conclusion, this study has shown that the conjugates exhibit markedly different systemic disposition profile than the control. The total body clearance of the drug associated with the conjugates decreases. This may indicate that although the liver is a major site for the retention of the conjugates, the elimination does not shift toward metabolism and the major route of elimination would still remain as urinary excretion. The ability of the dosage form to remain localized in the systemic circulation and the liver and its low concentration in the heart suggests its utility as a potentially effective delivery system for slow release and heart avoidance of doxorubicin.

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