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STUDIES OF THE STABILITY, PROTEIN BINDING AND
PHARMACOKINETICS OF DOXORUBICIN USING

A NEW ASSAY METHOD

BY

TYZZ-YUN CHEN

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

IN

PHARMACEUTICAL SCIENCES

UNIVERSITY OF RHODE ISLAND

1984

MASTER OF SCIENCE THESIS


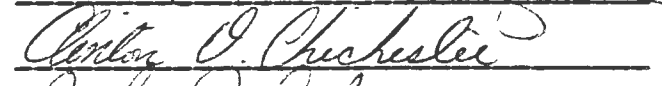
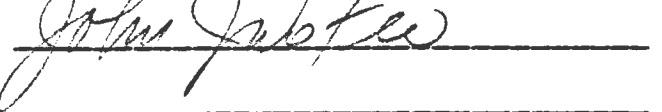
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Major Professor

DEAN OF THE GRADUATE SCHOOL

UNIVERSITY OF RHODE ISLAND

1984

ABSTRACT

Studies of doxorubicin pharmacokinetics have been bedeviled by the problems of low assay specificity and the possible degradation of doxorubicin and its metabolites during extraction. The purpose of this investigation was to provide a sensitive, selective, efficient and reproducible assay method for doxorubicin and its metabolites.

A C-18 reversed phase HPLC method was chosen to analyze the drug concentrations and the Sep-pak cartridges were used for sample preparation. The Sep-pak cartridge retained doxorubicin and its metabolites while interfering compounds (e.g., protein, cellular components) in the biological samples were eluted. Doxorubicin and its metabolites were then eluted with an acid-methanol mixture and concentrated in a water bath of 40° C. While plasma samples required no prior treatment before extraction, tissue samples were homogenized and released from binding to nuclear components by silver nitrate. The superiority of the Sep-pak method in sample preparation was established by comparing the efficiency, accuracy, processing time and the ease of operation with the conventional organic

extraction method.

The application of the assay method was tested in the plasma samples of human and rats, and tissue samples from rats. The results all showed a small variation and good agreement with the literature data. Pharmacokinetic profiles of these plasma samples were analyzed by AUTOAN and showed good correlation with those of literature and with each other. Plasma and kidney samples of the very young (2 months old) and the very old (2 years old) rats were analyzed but failed to observe any significant effect of age on doxorubicin pharmacokinetics.

During the development of the assay method, it was necessary to perform a study of doxorubicin stability to ascertain the best conditions for drug analysis. Doxorubicin showed to be more stable in acidic medium and the effects of pH have been quantified. Its stability in solution could also be influenced by the buffering agents used. The study of doxorubicin stability in plasma revealed that frozen plasma samples remained stable for 1 month and the thawing/freezing of these samples should be avoided.

Binding data obtained from the ultrafiltration method were unable to analyze due to high degree of binding to the

Diaflo membranes. This membrane binding property of doxorubicin not only caused an inconsistency among repeated experiments but also failed to provide an observation of the fraction bound. However, an ultracentrifugation method was performed and revealed that 0.7 fraction of doxorubicin was bound to 4 % albumin solution.

This study clearly demonstrated that the coupling of the Sep-pak method and the reversed phase HPLC system provided an efficient, sensitive, reproducible and accurate method for the pharmacokinetic studies of doxorubicin. This new method was also much easier to use than the organic extraction method. The stability studies indicated the suitable storage conditions for both plasma samples and doxorubicin solution during analysis. The binding data of 0.7 fraction bound of total doxorubicin was provided for future pharmacokinetic studies.

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I. INTRODUCTION

A. The Clinical Significance of Doxorubicin

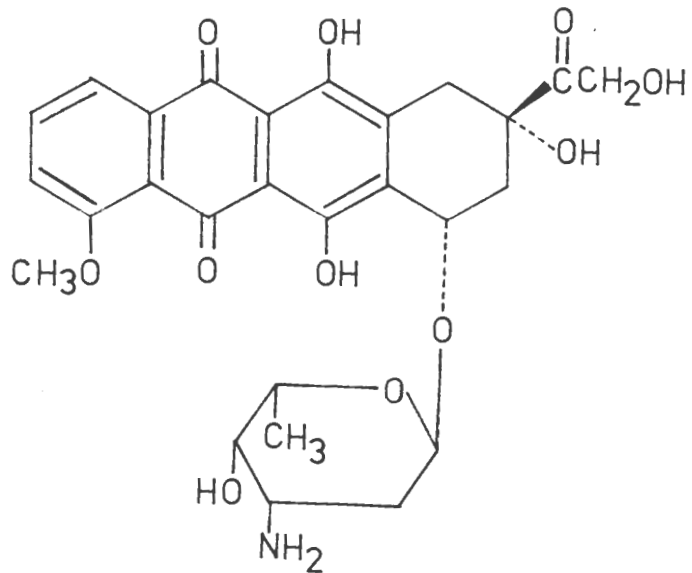
Cancer, perhaps the most feared of all diseases, has climbed to the second leading cause of death since 1976 (1). Study regarding the chemotherapy of cancer and the disease itself has been under extensive and vigorous investigation. Numerous chemical agents have been developed by prodigious efforts of more than a decade. Clinical results have demonstrated that the use of these agents has produced a significant cure rate of various neoplasms, particularly the rapidly proliferating cancers of childhood (2).

Unfortunately, most antineoplastic agents possess a therapeutic index that is virtually one, i.e., at an effective therapeutic level, toxic effects are also observed. Among the toxic symptoms, depression of bone marrow and toxicities toward gastrointestinal tract and mucous membrane are observed with the application of almost all the anticancer drugs (3). Another feature of these agents is the schedule-dependency which is the result of their effectiveness during certain phases of the cell cycle (4). Since active single agents are the requisite building block for a successful combined chemotherapy strategy, a

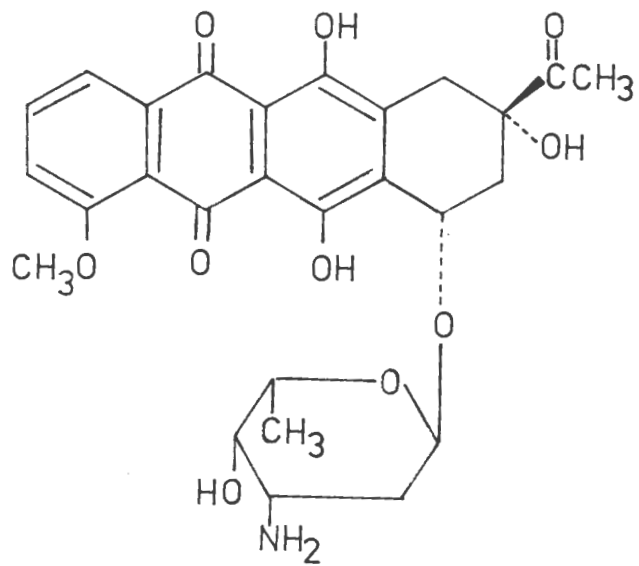
thorough understanding of the pharmacology and pharmacokinetics of these agents is imperative for their safe and effective use.

Doxorubicin was discovered by Arcamone et al. in 1969 and its chemical structure is shown in Fig. 1. It is produced by a mutant strain of Streptomyces peucetius (caesius variety), a microorganism that produces the closely related antibiotic, daunorubicin (also known as rubidomycin or rubomycin) (Fig. 1) (5). The attention focused on doxorubicin is mainly due to its efficacy against a broad spectrum of tumors while its parent compound, daunorubicin, is confined principally to the treatment of acute nonlymphocytic leukemia (6-10). In a report of cancer therapy protocols by the National Institution of Health in 1981, doxorubicin was employed in almost 40% of 1106 clinical protocols (11). With consideration of the progress in countries other than United States, it is impossible to overestimate the size of the ongoing clinical research of doxorubicin (12).

The clinical usefulness of doxorubicin in breast cancer, pediatric solid tumors, osteosarcoma, soft tissue sarcoma, Hodgkin's and other malignant lymphoma attests to an unprecedented selectivity for cytotoxicity of neoplastic



1.a



1.b

Figure 1. The chemical structure of doxorubicin (1.a) and daunorubicin (1.b)

cells (13). However, normal cells are not exempt from their toxic actions. Toxic effects such as alopecia, nausea, stomatitis and bone marrow depression, common in most antineoplastic agents, are generally manageable and reversible. However, a frequent irreversible and often fatal congestive heart failure, a dose and schedule related consequence of treatment, seriously restricts the clinical usefulness of doxorubicin (6-10,13,14). Although studies have supported the intravenous administration by an intermittent single bolus dose and a cumulative dose of less than 550 mg/m² is generally considered safe (10,13,14). These guidelines, based on statistical rather than pathophysiological and pharmacokinetic principles, often lead to underutilization of doxorubicin (14). To optimize the therapeutic benefits of doxorubicin, study of its pharmacology, toxicities and pharmacokinetics should be further engaged.

B. An Assay Method for Pharmacokinetic Studies

Although doxorubicin has been under investigation for more than 10 years, a detailed and accurate pharmacokinetic model is not yet developed. It would be advantageous to ascertain which parameter or parameters are related to the pharmacological activity of doxorubicin, and most importantly, which parameter is pertinent to the hazardous cardiotoxicity.

Early studies have suggested extensive accumulation of doxorubicin in several tissues and a slow and variable clearance (15,16). Clinical studies have clearly indicated a dose reduction in patients with either hepatic failure or a history of cardiac complications (16). It is only rational that a detailed understanding of the pharmacokinetic profile of doxorubicin is needed to determine the dose and schedule of administration to patients on an individual basis. Since doxorubicinol (the major metabolite) possesses antitumor activity and the possible involvement of aglycones in cytotoxicity, it would be sensible to include these metabolites in this kinetic study. The relationship between doxorubicin and its metabolites is depicted in the biotransformation pathway shown in Fig. 2. Due to the fact that considerable amount of doxorubicin is not recovered from excreta and cannot be

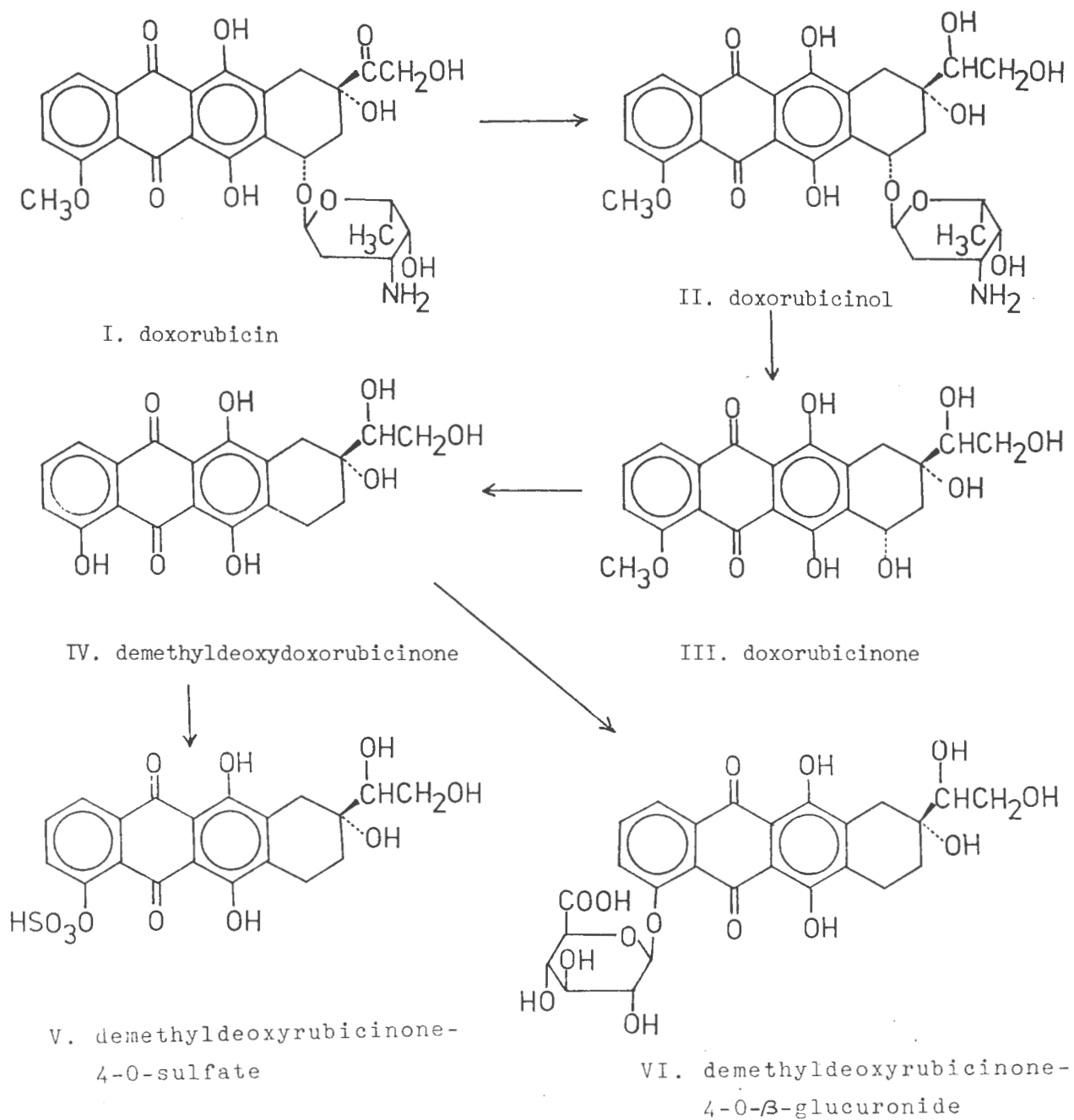


Figure 2. The proposed biotransformation pathway of doxorubicin

accounted for, it has been proposed that some unknown metabolites may be stored in the body (17-19) and the search of such compounds is encouraged.

1. The Problem

Confusion over the pharmacokinetic studies of doxorubicin arises mainly from the drawbacks of assay methods. The total fluorescence and the radiolabeled methods have shown no specificity, i.e., they are unable to differentiate doxorubicin and its metabolites (20-22). Additionally, it is reported that total plasma extractable fluorescence may be elevated due to the presence of certain steroids or bile acids causing interference, particularly at low drug levels (23).

RIA (radioimmunoassay) and GLC-mass spectrometry have demonstrated high specificity and sensitivity. However, they are expensive, time-consuming to use and GLC requires derivatization of the samples (24-27). At present, TIC and HPLC are the two methods generally used; this is partly due to the low cost of the procedures and the availability of instruments.

Quantitative TLC analyses are usually hampered by various problems such as non-uniform plate thickness,

difference in coating consistency, low sensitivity in detection and reproducibility (28). Although recent advances in methodology have improved these deficiencies (29,30), a standard graph must be prepared for each plate run together with the samples (31,32). In addition to these sensitive variables that influence reproducibility greatly, TLC method is accompanied with the falsely high levels of doxorubicinol (33). Reich et al. (1979) identified artifactual metabolites that chromatographed in authentic doxorubicin and aglycones bands on TLC plates, and they contributed 8 to 15 % to the metabolites levels (33). Isreal et al. (1978) performed a metabolism and excretion study with both HPLC and TLC methods, and found that at least one artifactual metabolite was seen only on TLC plates (30). Similar finding was identified by Benjamin et al. (1973), and they showed that the artifacts contributed 20% of the total fluorescence of extracted samples (15). Therefore, TLC method is considered an unreliable method unless care is taken to deal with these variable and artifactual metabolites levels.

Due to its flexibility and reproducibility, HPLC has become the optimal method for the analysis of many drugs; such as penicillin, cephalosporins and tetracyclines (34). As doxorubicin is concerned, HPLC is considered the method of choice owing to its low volatility and stability.

Various conditions have been developed and all have satisfactory specificity and sensitivity (24). However, reversed-phase chromatography is considered particularly suitable for the separation of a homologous series of compounds, as in this study, doxorubicin and its metabolites, whose chemical structure may differ one from another by only one functional group. In addition, this type of HPLC system elutes samples in reversed order of polarity which makes the detection of metabolites easier. A fluorescence detector is used in this study since it is more specific and sensitive than either U.V. or visible spectrophotometry (35).

As samples for pharmacokinetic studies are of biological origin, extraction becomes a necessity to eliminate the possible interference of components in biological fluids to compounds of interest (doxorubicin and its metabolites) and to chromatographic system. It is rare that samples do not require processing to prevent drugs binding to cellular components and to protect column packing and the detector of the HPLC system. This is usually done by homogenizing and extracting. The ordinary procedure for extraction is the conventional organic solvent extraction. Various organic solvents have been used with recovery rates of plasma samples ranging from 70 % to 100 % (31,36-40).

Better recoveries from plasma samples were seen mostly with cosolvent extraction, such as chloroform-methanol and chloroform-isopropanol mixtures or with acidified ethanol extraction. Although Eksborg (1978) has demonstrated, with chloroform-pentanol (9:1, v/v) mixture, that optimum extraction occurs around pH 8.3 for doxorubicin, pH 8.6 for doxorubicinol, and pH 8.0 for daunorubicin (41). Acidified ethanol (by hydrochloric acid of different strengths) showed a recovery rate of 85%, but the possible degradation by hydrolysis of the glycosidic bond and high fluorescence blank should be heeded (15,42,43). The other cosolvent extraction methods usually couple with alkalinizing the aqueous phase either by a buffer or sodium hydroxide solution (17,37,38,44,45). The recovery rates could reach as high as 100 %. However, variation caused by experimental conditions seems great since different values, 80, 84, 100 % of recovery were reported using the same method (17,44,45).

Successful application of this pH adjusted solvent extraction method to tissues and cells is seen in a smaller frequency, some even reported as low as 40 and 60 % of spiked amount is recovered (42,46). This loss of doxorubicin and its metabolites is attributed to the slow release of these compounds from cellular components (39). The strong and rapid binding ability of doxorubicin to DNA

has shown the association constant to be in the range of 0.37 to 11.61×10^6 M in in vitro experiments (47).

Schwartz (1973) demonstrated the capability of silver ions to release doxorubicin and daunorubicin from binding to DNA. Silver ions also precipitate protein, flavines and nucleotides, therefore considerably lower the fluorescence background (39). The employment of silver nitrite increased the recovery rate from 26 % to 100 % for doxorubicin, and from 55 % to 104 % for daunorubicin (39). The conventional solvent extraction method, however efficient, is a tedious method which requires adjusting pH, mixing, separating phases, and sometimes a second even a third extraction is needed. The development of a simpler method for extraction is one of the objectives of this study.

2. Theoretical Framework in Chromatography

HPLC (high pressure liquid chromatography) is a separation technique and in which quantitation, reproducibility and simplicity are made possible by the technical advancement in instrumentation. Chromatography is defined as a separation process that is achieved by exploiting the different intermolecular forces that are exerted on solutes when distributed between a mobile and

stationary phases. Those substances that are held more strongly in mobile phase pass through or from the system more rapidly than those that are held more strongly in the stationary phase. Thus, substances will move through or from the system in order of increasing forces that hold them in the stationary phase (48).

Although the separation process is exceedingly complex, it is possible to devise simple models and equations which closely approximate the separation. An understanding of these simple equations or relationships is essential when improvement of separation is needed. The ultimate goal of separation in HPLC is the achievement of the optimum combination of resolution of solutes, speed of elution, and economic use of pressure. In order to understand the relationship among these parameters, there are some fundamental terms that should be defined first (48,49).

dead time; void time; t_0 : the time that requires for an unretainable solute to be eluted from a column

dead volume; void volume; V_0 : the volume of eluent that requires for an unretainable solute to be eluted from a column

retention time; t_R : the time that elapse between injection and elution of a solute

retention volume; V : the volume of eluent that passes into the column during retention time

peak width; W ; the width of a peak at the baseline on a chromatogram

capacity factor; k' ;

$$k' = \frac{V - V_0}{V_0} = \frac{t_R - t_0}{t_0}$$

separation factor; selectivity; α ;

$$\alpha = \frac{V_2 - V_0}{V_1 - V_0} = \frac{t_{R2} - t_0}{t_{R1} - t_0}$$

theoretical plates; N ;

$$N = 16 \left(\frac{V}{W} \right)^2$$

resolution; R ; R_s ;

$$R = \frac{V_1 + V_2}{\frac{1}{2}(W_1 + W_2)}$$

$$\text{or } R = \frac{1}{2} \left(\frac{\alpha - 1}{\alpha} \right) N \left(\frac{k'}{1 + k'} \right)$$

The capacity factor, k' , is a measure of the degree of retention of a solute. If value of k' is higher than 8, this means the system wastes valuable analytical time. Value of k' smaller than 1 is also unfavorable due to potential interferences from nonretained peaks and early peaks of little or no analytical interest (49). The separation factor, α , is the net retention time ratio for two components. Fundamentally, α is equal to the ratio of equilibrium distribution coefficient, which depends solely on the molecular forces between the solute and the two phases, i.e., the mobile and stationary phases. N is the number of theoretical plates in plate theory and whose higher value means better separation and efficiency. For a good column, N should be in the reasonable range of 1,000 and 20,000 (50). Resolution, R , is simply a means of measuring the degree of separation of two compounds in a HPLC system. And resolution can be expressed in an equation which is the combination of selectivity (α), efficiency (N) and capacity (k') of a column. This version emphasizes three qualitative requirements for a good resolution; (1) solutes must be retained to different extents, i.e., $\alpha \neq 1$; (2) solutes must be retained, i.e., $k' \neq 0$; (3) the column must be equivalent to a minimum number of theoretical plates (N) (48-50).

These parameters, k' , α , N , R , are the guidelines to

judge whether a HPLC system is satisfactory or not. Improvement for the separation should be resolved from the basic Van Deemter equation which explains how factors in stationary and mobile phases influence the efficiency of a HPLC system (48,49). However, successful separations can be carried out only by careful experimentation preceded by shrewd planning.

Among the various modes of HPLC (IL, IS, GPC), reversed-phase chromatography has some distinct advantages as it is less likely to be deactivated, can efficiently separate both polar and ionic molecules, and it provides more freedom in choice of eluents. These advantages also act as a bonus in pharmacokinetic studies since highly polar materials are present in body fluids. Another advantage is the elution of solutes in reverse order of polarity which makes the detection of metabolites, usually polar than the parent compound, much easier. Besides, the handling of aqueous mobile phase is less hazardous than the organic one (48-50). However, commercially available reversed stationary phase sometimes may exhibit lower efficiency and lower loading capacities than their parent silica gel substrates (48). It is of interest to see whether the application of doxorubicin analysis in both reversed-phase and normal phase liquid chromatographies shows any qualitative and quantitative differences. If no

significant difference is detected in this study, reversed-phase HPLC stands as a favorable choice for the pharmacokinetic study of doxorubicin.

C. Stability Study

1. Purpose of This Study

Several fluorometric procedures for the analysis of doxorubicin have been reported (31,36-40), but little attention is given to its stability. It is very important to avoid artifacts caused by degradation prior to the quantitative determination of doxorubicin and its metabolites.

The solid products, either as doxorubicin hydrochloride salt or as a lyophilized mixture of doxorubicin hydrochloride and lactose, are stable if stored in dry and well closed containers at room temperature (51). The stability of aqueous solution of doxorubicin varies with temperature, pH and buffering agents (24,51). However, this study examines the effects of pH on the stability of doxorubicin since only one buffer system is used and the experimental conditions seldom suffer any drastic change of temperature in the whole process.

The same attention should be given to the handling of biological samples. Whole blood, owing to the enzymes it contains, exhibits metabolic activity toward doxorubicin. The plasma doxorubicin concentration of whole blood samples

decreased drastically during the first hour of incubation at 37°C (52). This effect is probably due to the cellular uptake of doxorubicin, as no detectable amounts of doxorubicinol appeared in the plasma fraction during this period of time. Small amount of doxorubicinol did appear in the plasma fraction when the whole blood samples had been incubated for more than 1 hour (52). In order to correlate the plasma levels with the concentrations of drug and metabolites in the circulating blood within the patients, reduction of this metabolic activity is desirable.

Formation of metabolites was reduced by storing the blood samples at lower temperature (4°C), by the addition of glutamate dehydrogenase or sonication (53). Though immediate sonication after the withdrawal of blood samples from patients has been suggested (53), it has been shown that poor recovery (70 %) resulted from this treatment (52). Since doxorubicin is not metabolized in cell-free plasma samples (54), the recommended procedure is immediate cooling of blood samples after withdrawal from patients and plasma fractions separated within 6 hours (52).

Though the usual procedure for handling plasma and tissues is to immediately deep freeze the samples until analysis (31,36-40), it has been demonstrated that there is

a decrease in doxorubicin concentration during storage of the frozen plasma samples (52). Eksborg et al. (1979) attributed this decrease in concentration to be the result of a change in the plasma matrix. This conclusion came from the observation that the amounts of precipitate in the thawed plasma samples increased with increasing storage time. It is likely that doxorubicin is adsorbed on the precipitate, which in turn decreased the degree of extraction into an organic phase (52). If this is the cause of decreased concentration in frozen sample, it is hoped that the Sep-pak method (the new method developed in this study) could overcome this flaw.

Since no direct contact between the plasma and organic solvent is present in this Sep-pak method, the possibility of C-18 bonded-phase silica stripping doxorubicin from its adsorbent is high. Additionally, the effects of the number of freezing/thawing cycles and of the storage time on doxorubicin concentration of frozen plasma samples is under investigation. The comparison of these two factors by freezing and thawing frozen plasma samples at different time intervals could determine the optimal storage condition. If the storage time plays a major role, it would be advisable to shorten the storage intervals between freezing/thawing. Otherwise, the repeated phases of freezing and thawing should be avoided as often as possible.

2. The pH-dependent Ionization of Doxorubicin

The evidence of enhanced stability of many drugs when the hydrogen-ion concentrations are maintained within a narrow range of pH, as well as of progressively decreasing stability when the pH departs from the optimum range, is abundant (24,41,51,55). Stability of a chemical may result from gain or loss of a proton by a substrate molecule, which reduces the reactivity of the molecule. In aqueous solution, instability may arise through the catalytic effect of acids or bases; the former by transferring a proton to the substrate, the latter by accepting a proton (55).

That doxorubicin is stable in acidic media and unstable in more alkaline ones is well known. This phenomenon results from the different ionized forms exist in solutions of different concentrations of hydrogen ion. These ionized forms not only exhibit different characteristics in stability, they also have different electronic absorption spectra. An ionization diagram postulated by Sturgeon and Schulman is presented in Fig. 3, which shows the relationship among these ionized species (56).

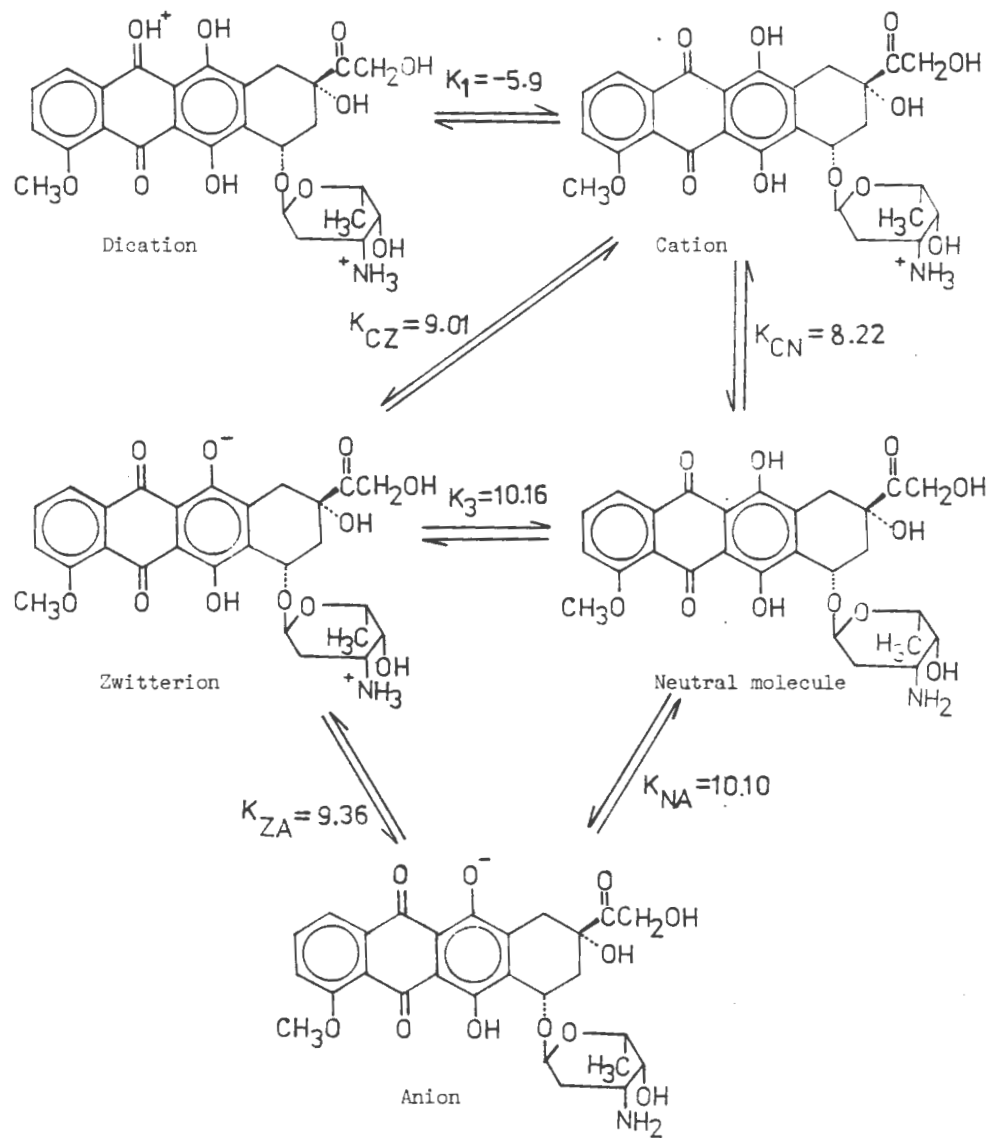


Figure 3. The protolytic equilibria of doxorubicin

From the listed dissociation acid constants in Fig. 3, it is observed that doxorubicin has several ionizable functional groups which are of similar acidity (56). Therefore, overlapping protolysis and equilibria are possible. There is an indication that three or more absorbing species are present in the solutions of pH 7-12 region because no distinct equivalence-point region is shown in titration curves (56,57). The acid dissociation constant of the monocation to form the neutral molecule is 8.22, which implies that a significant amount of doxorubicin is present as neutral form in solution slightly above this pH value. This coincides with the fact that maximum distribution of doxorubicin in organic phase occurs at alkaline media (pH 8.3) (41,56). Due to the existing of more than one ionized species in pH 7-12, the degradation process which happens much faster in strongly alkaline media may be due to the instability of one or more of the ionized species. It is hoped that from this study of the effects of pH on doxorubicin stability, more information regarding the relationship between ionization and stability may be found. It should be noted that the pKa values listed in Fig. 3 are only approximate since self-association of doxorubicin influences the data from one report to another (57).

In developing method of analysis for doxorubicin,

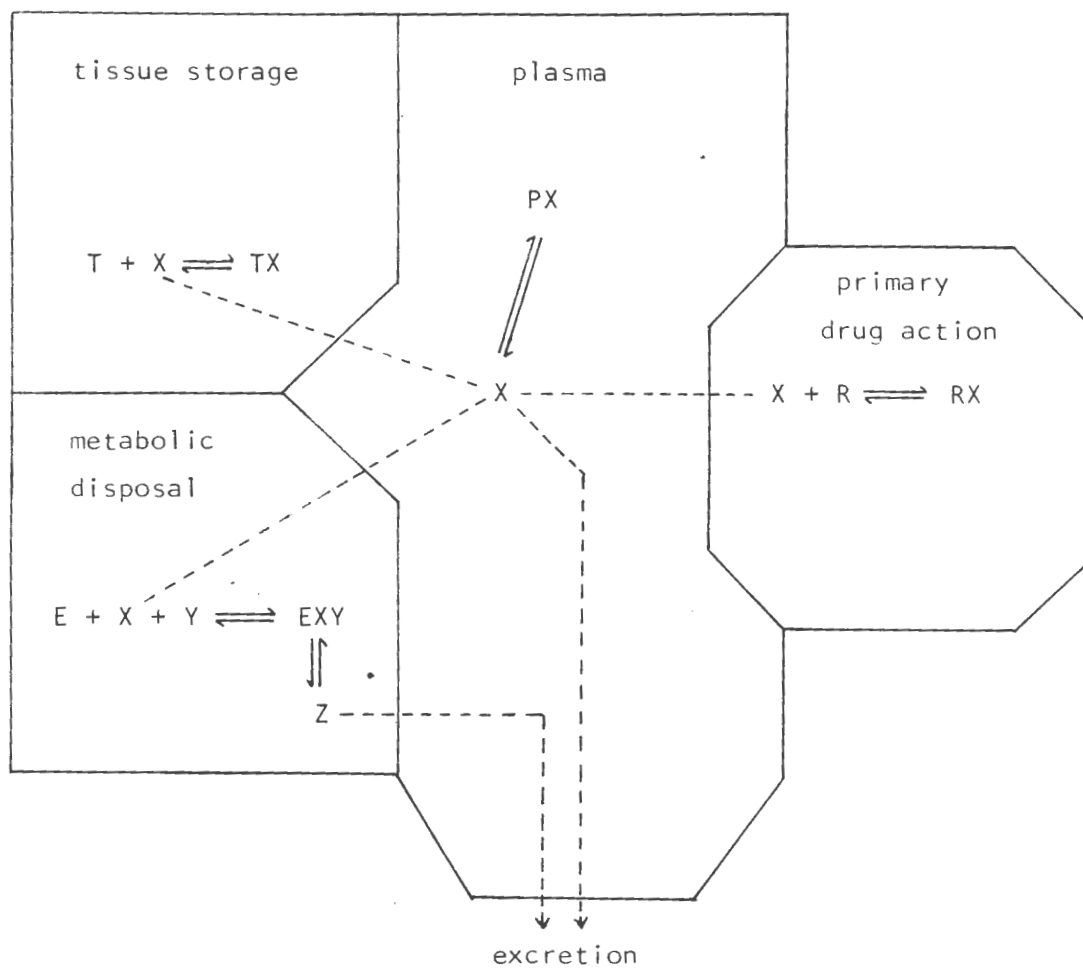
careful choice of pH and the analytical wavelength is necessary. The prevalence of only one emitting species having intense fluorescence in the pH 1-7 region suggests that the fluorescent analysis of doxorubicin should be carried out in acidic solution (56). The information of the ionization of doxorubicin also provides the rational basis for alkalinizing aqueous phase during extraction process.

D. Protein Binding Study

1. Scope and Purpose of This Study

Many drugs interact with plasma or tissue protein to form a drug-protein complex. Complexation of a drug with protein, i.e., drug protein binding, can influence the therapeutic, pharmacokinetic and toxicologic actions of the drug (58). Only free or unbound drug can pass through cellular membranes and reach the drug receptors or become eliminated (58,59). This relationship is shown in Fig. 4. However, only binding of drugs to plasma proteins has been extensively studied primarily because the plasma is readily accessible to sampling, can be easily separated into its constituent macromolecules, and is easily quantitated. Tissue binding has none of these advantages and, as a result, knowledge of the qualitative and quantitative aspects of the binding of drugs to tissue components is poorly understood (59). Wagner (60) and Gillette (61) have pointed out that tissue binding may be much more important pharmacokinetically than plasma binding. Owing to the difficulties to perform such a study, therefore, study of the plasma binding is the main goal of this study.

The major component of plasma protein responsible for drug binding is albumin, a protein molecule with a



Note: The size of each compartment has no physiological meaning

Figure 4. The postulated influences of plasma protein binding upon primary drug action

molecular weight of 69,000. Albumin is synthesized by liver and has a half-life of about 17 to 18 days in plasma (147,148). In the body, despite the large molecular weight, albumin is not exclusively retained in the plasma, but also distributed extravascularly into skin, muscle, liver, spleen, etc. (59). Normally, the albumin concentration is maintained at a relatively constant level within the plasma compartment at about 3.5-4.5 % (w/v) or $5.0-6.5 \times 10^{-4}$ M (58,59). The maintenance of a somewhat constant intravascular mass of protein (in particular, albumin) is physiologically critical because circulating intravascular protein is the principal determination of plasma volume (58,59). The change in plasma volume and in the free drug levels induced by a change in albumin concentration may produce significant differences in pharmacologic effects providing the fraction bound of this compound is high (58).

Drugs bind to albumin by either a reversible or irreversible process. In the case of irreversible drug binding which occurs rarely, the drug is chemically activated and attached permanently to the protein, usually by covalent bonding. Thus, the permanently protein-bound drug is unavailable for therapeutic use (58). When drug protein binding is reversible, the binding is initiated by electrostatic forces and the resulting complex is further

stabilized by van der Waals forces, sometimes a configuration change may occur to stabilize the complex (61). Therefore, the protein-bound drug act as a depot, slowly releasing the active drug and replacing the free drug which is eliminated, i.e., there is an equilibrium state between free and bound drug (58,61). Thus, protein binding of a drug can influence the distribution and elimination of the compound itself as well as the duration and intensity of the pharmacologic effects (59). However, evidence exists that only in case of highly bound agents will binding be important in a practical sense (58,59,62).

Quantitative information regarding the fraction bound of doxorubicin that binds to albumin was, however, unavailable. Harris and Gross (1975) found that an extent of 50 % of doxorubicin was bound to rabbit and human plasma using the ultracentrifuge method (63). Chen et al. (1978) re-analyzed the data of Harris and Gross, and determined the fraction bound in the therapeutic plasma concentration range was 0.9 rather than 0.5 with no further explanation for such a change (64). These reports leads to a fact that an accurate and detailed quantitative information regarding protein binding of doxorubicin is needed. Since the in vitro experiment should be performed in a slightly alkaline medium and the long period of time, 13 hours, is required to perform such a study using ultracentrifuge method (63).

It appears that the stability factor of doxorubicin should be considered. This study of doxorubicin binding kinetics in vitro can yield information that is valuable in the pharmacokinetic study of doxorubicin.

2. Theory of Protein Binding

The kinetics of reversible drug-protein binding can be described by the following equation (58,62) .

$$r = \frac{K \cdot C_f}{1 + K \cdot C_f} \quad (2.1)$$

r : molar ratio

K : association constant

C_f: the concentration of free doxorubicin

This equation describes the simplest situation, in which one mole of drug binds to one mole of protein in a 1:1 complex. The extent of the drug-protein complex formed is dependent upon the association binding constant, K.

However, protein molecules are quite large compared to drug molecules and may contain more than one type of binding site for the drug. If there is more than one type

of binding site and the drug binds independently on each binding site with its own association constant, then Eq. 2.1 expands to the following (58,62):

$$r = \frac{N_1 \cdot K_1 \cdot C_f}{1 + K_1 \cdot C_f} + \frac{N_2 \cdot K_2 \cdot C_f}{1 + K_2 \cdot C_f} + \dots$$

$$\text{or } r = \sum_{i=1}^n N_i \cdot \frac{K_i \cdot C_f}{1 + K_i \cdot C_f} \quad (2.2)$$

where the numerical subscript represent different types of binding sites, the K's represent the association constants and the n's represent the number of binding sites per molecule of albumin.

The values of the association constants and the number of binding sites can be obtained by various graphic methods. Scatchard plot is one of these graphic techniques which spreads data to give a better line for the estimation of the binding constants and binding sites. And due to this ability of spreading data points, Scatchard plot is the most common technique employed in protein binding studies. For a single binding site situation, the following is obtained (58,62)

$$r = N \cdot K \cdot C_f - r \cdot K \cdot C_f$$

$$\text{or } r/C_f = N \cdot K - r \cdot K \quad (2.3)$$

A graph constructed by plotting $r/(C_f)$ versus r yields a straight line with the intercept being $N \cdot K$ and the slope being $-K$. However, some drug-protein binding data produce Scatchard graphs of curvilinear lines, i.e., more than one type of binding sites exist (58). And non-linear fitting mathematical model using computer techniques is more suitable for such a complicated analysis rather than the direct estimation from the Scatchard plot. Using computer techniques, one can obtain best estimation for each N_i and each K_i (65). Also, the precision of the fitting procedure in the neighborhood of a least-squares solution can be examined (65). The development of such a computer program, however, is closely related to the binding method used to obtain raw data.

3. Binding Method and the Computer Nonlinear Fitting program

The degree of binding of drug molecules by protein can be estimated by using several methods such as dialysis, gel filtration, ultracentrifugation, and ultrafiltration. These methods yield similar, but not always identical results (62,66). Among them, a continuous ultrafiltration observes the binding behavior under various concentrations of drug with one single run. Thus, it provides a more accurate observation than the discrete data obtained by

other methods (67,68) .

Ultrafiltration is a process which separates dissolved molecules on the basis of molecular size. A moderate pressure (5 to 50 p.s.i) forces the solution and low molecular weight solutes through the thin membrane, but the passage of higher molecular weight solutes is hindered (66,67). Semipermeable dialysis membranes, e.g., Diaflo membranes, have been used as filters (67). In addition to the much higher efficiency of Diaflo membranes relative to those used in equilibrium dialysis, these membranes are also available with a spectrum of molecular size for retentivities.

Blatt et al. have demonstrated that the ultrafiltration method, coupled with the maintenance of fixed volume in the sample compartment during the run, performed protein binding studies in a manner similar to the conventional equilibrium dialysis, but without the protracted dialysis times common to the latter method (67). In a continuous process, it is possible to determine changes in binding from a single experiment (in a few hours) by taking aliquots of ultrafiltrate, carefully measuring the volume, and calculating the drug concentration (67,68). In comparison to dialysis method, continuous ultrafiltration has the advantages of obtaining a

series of data in a single run, separating the free drug over a short period of time, and the conservation of materials, particularly the expensive albumin. In addition, the membrane binding of the drug can be easily corrected by a blank run, i.e., without albumin present in the filtration cell (67,68).

A series of data regarding molar ratio (r) and free doxorubicin concentration (C_f) can be obtained by a continuous ultrafiltration method. And according to Eq. 2.2, a nonlinear computer program can be used to find the best estimates for n , each N_i and each K_i (65). This program also makes correction for the void volume of the system and the non-ideal behavior of the membrane. In comparison with the direct estimation from a Scatchard plot, a precision for the fitting procedure is provided and the probable error for each K_i is calculated.

With this mathematic binding model established for doxorubicin, the effects of the physiologic condition of a patient on protein binding can be predicted. If doxorubicin which has a small therapeutic index falls in the category of 'strongly bound', it would become necessary to consider protein binding as a vital parameter in the characterization of its behavior.

II. METHODOLOGY

A. Materials

1. Chemicals

Doxorubicin hydrochloride, Sigma Chemical Co.,
(D-1515, Lot 21F-0241)

Daunorubicin hydrochloride, Sigma Chemical Co.,
(D-4885, Lot 88C-0207)

Dimethyldichlorosilane, Pierce Chemical Co., (83401,
Lot 092581-82)

Hexane, Fisher Scientific Co., (H-302, Lot 793076)

Methyl alcohol, Burdick & Jackson Laboratories Inc.
(Lot AH749)

Phosphoric acid, 85%, Fisher Scientific Co., (A-260,
Lot 715056)

Silver nitrate, Mallinckrodt, (2160, Lot HN)

Acetonitrile, Waters Associates, Inc., (84935, Lot
081314)

Sodium lauryl sulfate, Ruger Chemical Co., Inc., (Lot
C713284)

Monobasic ammonium phosphate, General Chemical
Division, (1312, Lot K202)

Acetic acid, glacial, Fisher Scientific Co., (A-38,
lot 712081)

Chloroform, Waters Associates, Inc., (84939, AF612)

Magnesium chloride, Fisher Scientific Co., (M-33, lot
706396)

Methylene chloride, Fisher Scientific Co., (D-143, lot
711908)

Toluene, Fisher Scientific Co., (T-324, lot 742939)

Uracil, Eastman Kodak Co., (2504, lot A12A)

Hydrochloric acid, Mallinckrodt, (2612, lot KMBV)

Sodium hydroxide, Fisher Scientific Co., (S-318, lot
720859)

Albumin (human), fraction V power, Calbiochem-Behring
Corp., (12666, lot 903635)

Citric acid, Amend Drug and Chemical Co., (lot
26698M31)

Boric acid, Amend Drug and Chemical Co., (lot C611549)

Monopotassium phosphate, Fisher Scientific Co.,
(D-286, lot 722964)

Disodium phosphate, Merck, (74241, lot 62875)

2. Equipment and Supplies

Vortex-genie, Scientific Industries, Inc.

Sep-pak cartridges, Waters Associates, Inc.

Plastipak syringes, Fisher Scientific Co.

Sample vials and sample rack of Fisher Model 190

Sample concentrator, Fisher Scientific Co.

Tissue grinders, Potter-Elvehjem type, and telphon
pestles, Thomas Scientific Co.

Magni Whirl constant temperature bath, Blue M Electric
Co.

Vacuum pump, Model 0211-V36A, Millipore

HA membrane filters (0.45 μm), Millipore

FA membrane filters (0.5 μm), Millipore

μ Bondapak C-18 column, 3.9 mm (ID) x 30 cm, (10 μm),

Waters Associates, Inc.

Radial-pak B liquid chromatographic cartridge 0.8 mm
(ID) x 10 cm, (10 μm), Water Associates, Inc.

Pellicular media for HPLC, Octadecyl (C-18) group,
(30-38 μm), Whatman, Inc.

Model RCM-100 Module (of the radial compression
separation system), Waters Associates, Inc.

Model 6000A solvent delivery system, Waters
Associates, Inc.

Model 420 fluorescence detector, Waters Associates,
Inc.

Guard Column, 3.2 mm (ID) x 5 cm, Rainin Instrument,
Cc. Inc.,

WISP (Water Intelligent Sample Processor) 710B, Waters
Associates Inc.

Data Module, Waters Associates, Inc.

Expandomatic SS-2 pH meter, Beckman

Dynac II Centrifuge, Clay Adams.

Mettler Balance, type H6T, Mettler Instrument Corp.

Mettler Balance, type H16, Mettler Instrument Corp.

Torsion Balance, The Torsion Balance Co.

Series-parallel R-C combination box Model 1140,

Electric Instrument Co. Inc.

SC 102 Active filter, Analabs

Ultrafiltration unit, model M8, Amicon

Diaflo ultrafilters, PM10, Amicon

Centrifree, Amicon

B. Assay Method

Doxorubicin, its metabolites and daunorubicin (the internal standard) were extracted from biological samples by a new procedure developed to replace the tedious conventional organic extraction method. In this new method, Sep-pak cartridges are used to prepare protein free samples for HPLC analysis. These cartridges are small, self-contained and packed with liquid chromatographic separating materials optimized for sample preparation and cleanup procedure. Compounds of interest (doxorubicin, its metabolites and daunorubicin) can be retained in this chromatographic bed while materials such as proteins and nucleotides pass through. Compounds of interest can then be eluted with an appropriate solvent.

Biological samples of interest are human and rat plasma, and rat tissues (e.g., liver, kidney, lung, heart, and brain) which were kindly provided by Roger Williams General Hospital. Plasma, being a cell-free fluid, could be directly applied to Sep-pak cartridges whereas tissue processing required homogenization. Cells in tissue, upon homogenization, broke into a mixture of cellular components, such as nucleotides, proteins, ribosomes, mitochondria, etc.. When doxorubicin was exposed to such attractive substrates, especially nucleotides and

ribosomes, it was highly possible that doxorubicin (DCX) existed mostly as the DOX-DNA complex in the homogenate (39). This was further confirmed by the in vitro binding studies of doxorubicin and DNA, which showed a high association constant of $0.37 - 11.61 \times 10^6$ M (47).

The unsuccessful extraction of doxorubicin from tissue homogenates indicated that a stronger reaction was needed to dissociate the DCX-DNA complex rather than the simple partition phenomenon used in the organic extraction method (42,46). The use of silver nitrate proposed by Schwartz solved this problem by precipitating nucleotides and proteins and thus free doxorubicin from its binding sites (39). But in the case of plasma samples, the only possible substrate is albumin. The organic extraction method has been demonstrated to be sufficient to disrupt the binding forces between doxorubicin and albumin (31,36-40). Therefore, there are different approaches for handling samples, which are dependent on the origins of these samples, and will be further explained in the following procedures.

Human and rat plasma, and rat kidney samples were available for pharmacokinetic studies. Plasma samples from four cancer patients with various disease were assayed. Doxorubicin was administered as a single bolus dose in all

these patients and plasma samples were taken at different time intervals. Data were analyzed by AUTCAN to calculate the pharmacokinetic parameters. It would be of interest to see whether these parameters, such as half-life, peak concentration, volume of distribution, would be influenced by the disease state, age and sex of each patient.

Plasma and kidney samples of both young and old rats were assayed by the Sep-pak method. This study was inspired by the deeply concerned effects of age on pharmacokinetics. Pharmacokinetic studies in geriatrics is of substantial interest with the recognition that the aged constitute an increasing proportion of patient population (69-71). It is hoped that from this small scale study, some contribution may be made in this field. However, the results may be partial and incomplete since only the plasma and kidney samples were analyzed.

Plasma and kidney samples of both groups (the young and the old) were taken at 0.5, 1.5, 4, 10, 24, 50 hours after the tail-vein injection of 15 $\mu\text{g}/\text{Gm}$ of doxorubicin. There was one rat at each time interval per sample group (A,B,C,D) and 24 rats were used in this study. The age of the young rats (group A and B) were 2 months and those of the old rats (group C and D) were an average of 2 years. Samples were all frozen at -20°C until analysis. Data from

this study was also calculated by AUTCAN and compared between these groups.

1. Silanization of Glass Equipment

All glass equipment, with the exception of the injection syringe, was silanized before use by treatment with dichlorodimethylsilane (5 %, by volume) in hexane overnight, followed by washing with methanol.

In order to exhibit the effect of silanization, extraction was also carried out with the non-silanized glassware. Two sets of glassware (the silanized and the non-silanized) were used during extraction procedure, after the preparation of stock solutions of doxorubicin and daunorubicin.

2. Plasma Samples

0.1 ml of internal standard (daunorubicin) solution in an appropriate concentration was added to 1.0 ml plasma sample. Since doxorubicin concentration fell from 1,000 ng/ml to less than 100 ng/ml during a study, it was necessary to use two concentrations of daunorubicin solution, either 2 or 8 µg/ml, to make the most accurate and sensitive detection. After the addition of internal

standard, the sample was processed by the Sep-pak method.

3. Tissue Samples

Frozen tissues were thawed and samples ranging from 100 to 400 mg (dry weight) were taken. 0.1 ml of 8 µg/ml daunorubicin solution was added to the tissue and sample was homogenized in 2 ml of water employing a Potter-Elvehjem homogenizer. 0.3 ml of 50 % silver nitrate (freshly prepared, weekly) was then added to the homogenate and mixed well with the pestle. Finally, 2 ml of water were added to each sample.

Schwartz employed 0.1% sodium lauryl sulfate solution to facilitate the lysis of L 1210 ascites cell in the presence of silver nitrate (39). The effectiveness of sodium lauryl sulfate solution in tissue homogenates was also tested. This test was performed by adding 2 ml of 0.1% sodium lauryl sulfate to the silver-ion-treated homogenates. Additionally, the efficiency of pH 7.4 phosphate buffer to extract doxorubicin and related compounds (72) was investigated. This was done by homogenizing the sample in two portions of 4 ml phosphate buffer (pH 7.4) after the addition of the internal standard. Homogenates, obtained from respective procedures, were then centrifuged at 2,000 r.p.m. for 10

minutes. The clear supernant was reserved for extraction.

4. Extraction Methods

a. The Sep-pak method

To avoid adsorption of doxorubicin onto glass, plastic syringes were used in this procedure. The Sep-pak cartridge was first wetted by 5 ml of methanol, followed by 10 ml of water in order to remove residual methanol which may precipitate proteins and elute compounds of interest prematurely. The plunger was then removed from the syringe and sample was introduced with Sep-pak cartridge attached to the tip of syringe. For plasma samples, the introduction was simply a direct application. For tissue samples, it was the clear supernant of the centrifuged homogenates. After removing the non-retainable portions of sample, the Sep-pak cartridge was washed with 12 ml of water to remove any residual protein or silver ions. The retained compounds in the cartridge were then slowly eluted with 7 ml of acid-methanol (5×10^{-3} M phosphoric acid in methanol) into an evaporation sample vial.

The strong acidity of this acid-methanol mixture (pH 2.34) may promote the possible hydrolysis of the glycosidic bond of doxorubicin. Therefore, two drops of 20% NaOH in

methanol were immediately added to the individual eluent to adjust the pH to around 6. The pH adjusted eluents were then concentrated in a 40°C water bath until sample volume was approximate 1 ml (about 3 hours). Samples were then analyzed by an HPLC system consisted of a solvent pump, a C-18 chromatographic column, a fluorometer and a data acquisition system (Data Module, Waters).

b. The organic solvent extraction method

An existing organic extraction method was tested in order to determine whether extraction efficiency of the Sep-pak method was superior or not. 0.1 ml of 2 µg/ml daunorubicin solution was added to 1.0 ml of spiked plasma. Two drops of 0.1 N NaOH solution were then added to the sample to adjust the pH for maximum extraction. 4 ml of a chloroform and methanol mixture (4:1) was used to extract doxorubicin thrice. Extract was placed in an evaporation sample vial and concentrated in the same manner as previously described. Samples were then blown to dryness using nitrogen gas and redissolved in 1 ml of methanol for HPLC analysis.

c. Calculation of recovery rates

Three methods were used to measure the absolute amounts of doxorubicin recovered from extracts. The first method was the standard addition method which used the internal standard to compensate the quantitation differences caused by complex sample matrices. In this method, the processed sample from spiked plasma was chromatographed first. The sample was then spiked with a known amount of doxorubicin and quantitated again. The change in the peak height (PH) of the unspiked daunorubicin (IS) peak was used as an adjustment factor to correct for the concomitant sample dilution. The formula for the standard addition method are:

$$\text{adjusted PH of DOX} = \frac{\text{PH of IS before spike}}{\text{PH of IS after spike}} * \text{PH of DOX spiked}$$

$$\text{amount in the sample} = \frac{\text{amount spiked} * \text{PH of DOX unspiked}}{\text{adjusted PH of DOX} - \text{PH of DOX unspiked}}$$

The recovery rate could then be calculated as following:

$$\text{recovery rate} = \frac{\text{amount in the sample}}{\text{amount spiked before extraction}} * 100\%$$

The second one was the direct method whose major difference was the addition of the internal standard after concentration of the extracts. The final addition of daunorubicin was to correct the sample volume differences. The peak heights (PH) of doxorubicin and daunorubicin were measured against the standard doxorubicin and daunorubicin solution of the same concentration. Recovery rates could be calculated as the following equations:

$$\text{adjusted PH of DOX} = \frac{\text{PH of DOX} * \text{PH of IS in STD}}{\text{PH of IS}}$$

$$\text{recovery rate} = \frac{\text{adjusted PH of DOX}}{\text{PH of DOX in STD}} * 100\%$$

where DOX stands for doxorubicin and STD represents standard solution.

The third one was the elution method which compared doxorubicin concentration of the unconcentrated eluent from a Sep-pak cartridge with that of a standard solution. This was done by assaying the drug concentration in the eluent from a spiked plasma or tissue sample before concentration in a water bath. The standard solution was prepared by adding the same spiked amount into the same volume (as that of the eluent) of acid-methanol mixture. And the recovery rate was calculated by the following equation.

$$\text{recovery rate} = \frac{\text{conc of DOX in the eluent}}{\text{conc of DOX in STD}} * 100\%$$

While the extraction efficiency of the Sep-pak method was calculated by all three methods, that of the organic solvent extraction was only calculated by the direct method.

5. High Performance Liquid Chromatographic System

a. Chromatographic system for analysis

Various liquid chromatographic systems have been developed for the analysis of doxorubicin and its metabolites (15,24,34,35). However, in the present study, reversed-phase chromatography was used for all assays owing to the advantages described before.

A 3.9 mm (ID) x 10 cm stainless steel column, packed with 10 μ m particle-size octadecyl-silica (μ Bondapak C-18, Waters) was used. An isocratic mobile phase consisted of 600 ml of methanol and 400 ml of 0.01 M $\text{NH}_4\text{H}_2\text{PO}_4$ solution (to the latter, 5 ml of acetic acid was added). This mobile phase (final pH=4) will be referred to as mobile phase 1, the methanol and monoammonium phosphate solution

(60:40) mixture. The flow rate was 1.0 ml/min and resulted in a pressure of 1,500 p.s.i.. The mobile phase was degassed by filtering through FH type filter (0.5 μ m, Millipore) under vacuum. All separations were performed at ambient temperature. Samples were injected using an automatic sample processor (WISP, Waters).

The detection unit consisted of a filter-type fluorescence detector (Model 420, Waters) and a SC 102 active filter (Analab). The fluorescence detector measured fluorescent luminescence with an emission filter of a band of wavelengths around 254 nm and an excitation filter that cut off wavelengths greater than 495 nm. The fluorescence detector was equipped with a 10- μ l flow cell. The SC 102 active filter maintained peak amplitudes while reducing the baseline noise with no signal distortion. Chromatograms were recorded and peak height areas (referred to as peak heights in the following text in order to distinguished from total peak area) were integrated by a data acquisition system (Data Module, Waters).

All sample injection was performed by the automatic sample processor (WISP, Waters). Due to the wide physiological range of doxorubicin concentrations (15,25,31,44,73-75), the adjustment of the injection volume of sample was necessary (Appendix). There was also a

lowest detection limit which required the signal to noise ratio to be larger than 5. This restriction in detection limit required that the concentrations of daunorubicin changed correspondingly to those of doxorubicin (Appendix). The automatic sample processor responded accurately to both adjustments.

b. Measurement of the efficiency of separation

The separation efficiencies of a chromatographic system can be easily described by parameters such as capacity factor (k'), selectivity (α), theoretical plates (N) and resolution (R). In order to measure and calculate these parameters, the void volume of the system should be determined first. The void volume of C-18 column (μ Bondapak, Waters) was measured by injection of non-retainable uracil with mobile phase being a mixture of acetonitrile and water (60:40). The retention times of doxorubicin, daunorubicin, doxorubicinone (the aglycone) and doxorubicinol (the major metabolite) were determined by using the same column but with mobile phase 1, the methanol and monoammonium phosphate solution (60:40) mixture. Then the capacity factors (k'), number of effective plates (N), selectivities (α), and resolutions (R) of CR among these four compounds were calculated.

Daunorubicin and doxorubicin were purchased from Sigma and used as received. Doxorubicinone can be synthesized by hydrolysis of doxorubicin in 1 N HCl at 100°C for 30 minutes (89). But doxorubicinol was obtained from the tissue samples. Due to the lack of doxorubicinol standard, it would be necessary to verify its existence by comparing literature data with more than one chromatographic systems. In addition, it would be of interest to measure the efficiencies of these systems and to make sure that the choice of the analysis system (Sec. 5a) did not suffer in efficiency and sensitivity. The second chromatographic system consisted of the same C-18 column (µBondapak, Waters) and a mobile phase prepared from 0.01 M phosphoric acid in acetonitrile solution. Various fractions of acetonitrile were tried to obtain the best separation profile and the highest sensitivity. The flow rate was 1.0 ml/min and produced a pressure of 1,000 p.s.i. The void volume was the same as with system 1 since the same column and tubing were used.

The third system consisted of a normal phase, Radial-pak B chromatographic cartridge (10 µm, Waters), and mobile phase being a mixture of chloroform, methanol, glacial acetic acid and 3.0 mM magnesium chloride solution (720:210:40:30). This Radial-pak cartridge was properly pressured in the Model RCM-100 Module. The flow rate was

2.0 ml/min with pressure being 1,000 p.s.i.. The retention times of all four compounds (doxorubicin, doxorubicinone, doxorubicinol and daunorubicin) were measured with the same detection unit and recorder. The void volume of this system was obtained by injection of the non-retainable toluene with mobile phase being methylene chloride. Other parameters like K' , α , N , R , were also calculated. As for measuring the sensitivity of each system, samples of the same concentrations of doxorubicin (500 ng/ml) and daunorubicin (1 μ g/ml) in respective mobile phase were injected and the peaks measured.

All mobile phases were degassed by the same vacuum pump method and separations were performed at ambient temperature.

C. Method for Stability Studies

1. Stability of Doxorubicin in Solutions

The stability of doxorubicin was measured in citrate - phosphate - borate buffers with pH ranging from 2.00 to 11.90. Because all spiked samples were prepared from aqueous solutions of doxorubicin, its stability in water was also measured. Additionally, the stability test was also performed in acid-methanol (eluent in Sep-pak method), mobile phase 1 (the 0.01 M monoammonium phosphate solution-methanol (60:40) mixture), and pH 7.0, 7.4 phosphate buffers. The phosphate buffers was included in this test because it was used in the protein binding studies (pH 7.4) and to detect the effects of changing the buffering agents.

Solutions of 1 $\mu\text{g}/\text{ml}$ of doxorubicin in citrate - phosphate - borate buffers, water, acid-methanol, mobile phase 1 and phosphate buffers (pH 7.0 and 7.4) were analyzed by HPLC at appropriate time intervals (dependent on the rate of degradation). The mobile phase for this study was 0.01 M phosphoric acid in 40 % acetonitrile solution. All samples were prepared in triplicate and analyzed twice.

2. Stability of Doxorubicin in Plasma

The effects of storage time and the number of freezing/thawing cycles on the stability of doxorubicin in plasma were investigated. Frozen spiked plasma samples of 500 ng/ml doxorubicin were thawed at room temperature at intervals of 1, 3 and 5 days. Samples that were thawed after staying frozen for 10, 30 and 50 days served as the control groups. 0.1 ml of daunorubicin solution (7.5 µg/ml) was added to 1.0 ml of a thawed sample and Sep-pak extraction method was used to extract both compounds from plasma. The concentrated eluents were analyzed by a reversed-phase HPLC system while mobile phase was prepared from 0.01 M phosphoric acid in 40 % acetonitrile solution. All samples were prepared in triplicate and analyzed thrice.

Eksborg et al. proposed that the degradation may be caused by the adsorption of doxorubicin onto the precipitate of thawed sample (see Introduction C.1, 52). Therefore, a visual check-up for the appearance of thawed sample was also performed.

D. Methods for Protein Binding Studies

1. The Ultrafiltration Methods

Ultrafiltration was performed with an Amicon Model M8 ultrafiltration unit. A PM 10 Diaflo ultrafilter was used to retain particles of molecular weights larger than 10,000. Before performing a continuous ultrafiltration, a direct (i.e., a 'wash-out') experiment was carried out first to determine the appropriate doxorubicin concentrations used in a continuous method.

The 'wash-out' procedure entailed the filtration cell with 8 ml of doxorubicin solution of known concentration in 4 % albumin solution (in pH 7.4 phosphate buffer). Ultrafiltration was commenced with a stir bar connected to the cap of the filtration cell. Agitation was needed to maintain uniform bulk composition in this compartment and to prevent polarization or caking out of high molecular weight species against the membrane surface (67,68). A pressure of 10 p.s.i. of nitrogen gas was used to push the free (unbound) doxorubicin through the Diaflo membrane. Various concentrations of doxorubicin were tried until a significant amount of doxorubicin in ultrafiltrate was detected. This method also provided a rough estimation of the fraction of doxorubicin bound by albumin. A blank

'wash-out' experiment was simultaneously carried out to quantitate the fraction of doxorubicin that bound to the Diaflo membrane itself. Therefore, two sets of direct ultrafiltration experiments were performed; one with doxorubicin and 4 % albumin solution in the filtration cell, and the other (the blank run) with only doxorubicin solution. However, it should be pointed out that no more than 10 % of the fluid volume in the filtration cell (i.e. 0.8 ml) was allowed to collect otherwise the resultant change of the albumin concentration may significantly affect binding.

From these 'wash-out' experiments, the concentrations of doxorubicin used in the continuous ('wash-in') method could be determined. In this 'wash-in' experiment, 50 ml of doxorubicin (5, 10, 15 $\mu\text{g/ml}$ respectively) in pH 7.4 phosphate buffer was placed in the reservoir cell and the filtration cell was filled with 8 ml of 4 % human albumin in pH 7.4 phosphate buffer. A pressure of 10 p.s.i. was exerted on the reservoir cell to push doxorubicin into the filtration cell. At the same time, the same volume of liquid was pushed out as the ultrafiltrate. To prevent degradation of doxorubicin caused by the high pH (pH 7.4), six drops of 15 % HCl solution was immediately added per gram of the weighed ultrafiltrate to lower the pH to around 5. The pH adjusted ultrafiltrates were again weighed to

compensate for this dilution effect. The processed ultrafiltrates were analyzed using the HPLC system as previously described but with a mobile phase of 0.01 M phosphoric acid in 35 % acetonitrile solution.

The Diaflo membranes were highly stained by doxorubicin, indicating substantial membrane binding. Correction for membrane binding was accomplished by a blank 'wash-in' experiment with the same concentration of doxorubicin in the reservoir cell and no albumin (only phosphate buffer) in the filtration cell. The ultrafiltrate thus collected was also treated in the same manner as previously described. The same blank experiment was also performed in the 'wash-out' method to correct both membrane binding and void volume.

2. The ultracentrifugation method

An ultracentrifugation method was performed to see if the binding data did vary from one method to another as others have indicated (154,156). This was done by using the disposable Centrifrees (Amicon) and only one concentration of doxorubicin was covered due to the limited availability. 5 µg/ml of doxorubicin in 4 % albumin solution (in pH 7.4 phosphate buffer) was added to the sample reservoir of a Centrifree. The Centrifree was then

spun at 2,000 r.p.m. for 20 minutes. The filtrate thus obtained was weighed, neutralized with 15 % HCl solution and weighed again. The pH adjusted filtrate was assayed chromatographically and correction for the dilution effect of neutralization was made. A blank run, with no albumin in the sample reservoir, was carried out to determine the nonspecific adsorption of doxorubicin to the membrane.

III. RESULTS AND DISCUSSION

A. The Assay Method

1. High Performance Liquid Chromatographic Systems

The efficiencies and sensitivities of three HPLC systems were investigated and the results were shown in Table 1. Chromatographic parameters such as capacity factor (k'), selectivity (α), number of effective plates (N), and resolution (R) were calculated for each system. Comparison was made and the suitable application of each system was indicated.

a. The phosphoric acid-acetonitrile system

This mode of HPLC system consisted of a μ Bondapak C-18 column (Waters) and a mobile phase of 0.01 M phosphoric acid in various fractions of acetonitrile solution. The highest sensitivity could be obtained when the fraction of acetonitrile was over 35 % (Table 1). Although separations among compounds of interest (doxorubicin, its metabolites and daunorubicin) were improved by lowering the fraction of acetonitrile, the sensitivities were simultaneously lesser. Due to this phenomenon, it was suggested that suitable alteration of the acetonitrile fraction be made to meet the

HPLC system	relative absorbance of DOX (%)	relative absorbance of DAU (%)	specified uses
40%CH ₃ CN	100.00	100.00	for stability studies
35%CH ₃ CN	100.00	100.00	for protein binding studies
32%CH ₃ CN	63.73	69.38	no specific uses, the separation of doxorubicinol was not good
30%CH ₃ CN	53.26	56.85	no specific uses, the separation of doxorubicinol was not good
65%MeOH	36.31	42.80	no specific uses, the separation of doxorubicinol was good enough
60%MeOH	30.74	31.98	good for assay of the biological samples
21% CHCl ₃	34.23	69.29	the capacity factors were too small for all compounds

- *1 This refers to a reversed phase HPLC of a C-18 column and mobile phase being 0.01M H₃PO₄ in acetonitrile solution. The percentage of acetonitrile is assigned.
- *2 This referred to a reversed-phase HPLC of a C-18 column and mobile phase being 0.01 M NH₄H₂PO₄ solution and methanol mixture. The percentage of methanol is assigned.
- *3 This referred to a normal-phase HPLC of Radial-pak E column and mobile phase consisted of MeOH, CHCl₃, MgCl₂ solution, glacial CH₃COOH (21:72:4:3).
- *4 This measurement was performed with solutions of DOX (doxorubicin 500 ng/ml) and DAU (daunorubicin 1µg/ml) in respective mobile phase. The variation was smaller than 5 % and therefore is omitted from this table.

Table 1. The relative absorbances and separation conditions for HPLC systems

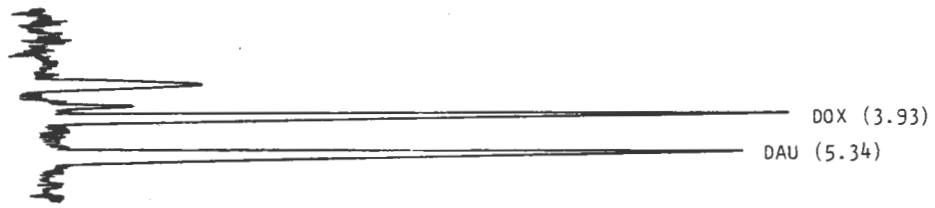
requirements of each type of doxorubicin analysis.

Mobile phase containing the highest fraction of acetonitrile, i.e., 40 %, was most suitable for the analysis of doxorubicin in stability studies (Fig. 5). Due to the low interferences in plasma and buffers, good separation and high sensitivity were observed during the whole process. The retention times of doxorubicin and daunorubicin were 3.93 and 5.34 minutes respectively. The minimal requirement for a good separation in HPLC is to set up conditions so that the selectivities fall into a range of 1.05 - 2.00, and resolutions are greater than 1.5. The selectivity in this system between the nearest interference peak and doxorubicin in spiked plasma was 1.59 and that between doxorubicin and daunorubicin was 2.28 (Fig. 5.b). The resolution was 1.64 between the former set of compounds and 3.52 between the latter. The application of this mobile phase was also very economic in time and solvents. The analysis time of each sample was 5 minutes for stability tests in buffers and 7 minutes in plasma samples.

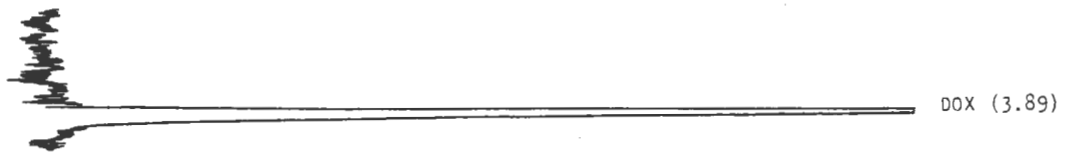
The mobile phase containing 35 % acetonitrile was used for all protein binding studies. As shown in Fig. 5, some degree of interference did exist for the first few fractions of ultrafiltrates collected. And this mobile phase could effectively separate these interferences from



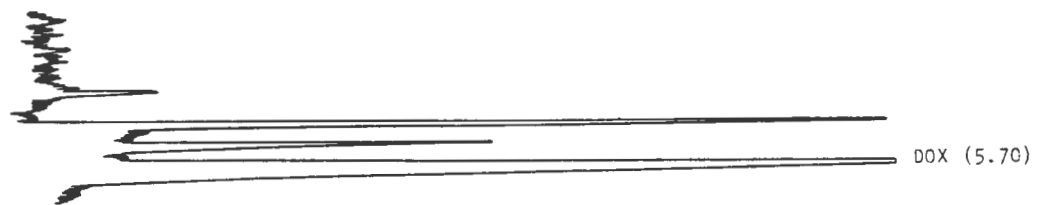
a. blank plasma sample in 40 % acetonitrile



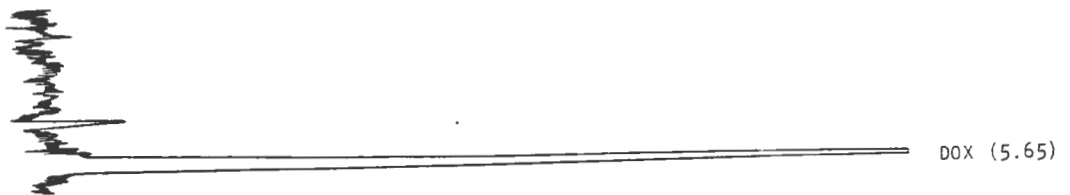
b. spiked plasma sample in 40 % acetonitrile



c. spiked buffer sample in 40 % acetonitrile



d. ultrafiltrate sample in 35 % acetonitrile



e. spiked sample of the neutralized phosphate buffer in 35 % acetonitrile

Figure 5. The chromatograms of doxorubicin and daunorubicin in the phosphoric acid - acetonitrile system

doxorubicin without losing any degree of sensitivity (Table 1). The retention time of doxorubicin was 5.70 minutes. The selectivity between the last peak of interferences and doxorubicin was 1.39 and the resolution was 1.45 (Fig. 5). The analysis time of each fraction of ultrafiltrate was 7 minutes.

Void volume for the above calculation of selectivities were measure by injection of uracil with a mobile phase of 60 % acetonitrile solution. The void volume thus obtained was 2.83 ml. While these mobile phases were efficient enough for stability and protein binding studies, it was not until the fraction of acetonitrile was as low as 30 % that the detection of doxorubicinol in tissues samples was possible. The chromatograms of the liver samples in this mobile phase was shown in Fig. 6. The peaks of doxorubicinol and doxorubicinone were barely recognized. The chromatographic parameters of the compounds of interest were listed in Table 2. The selectivity and resolution between the last peak of interferences and that of doxorubicinol were 1.12 and 0.52 respectively. The resolution was also poor between doxorubicinone and daunorubicin. This mobile phase was not applicable for the assay of tissue and plasma levels.

The chromatographic data obtained by Eksberg showed

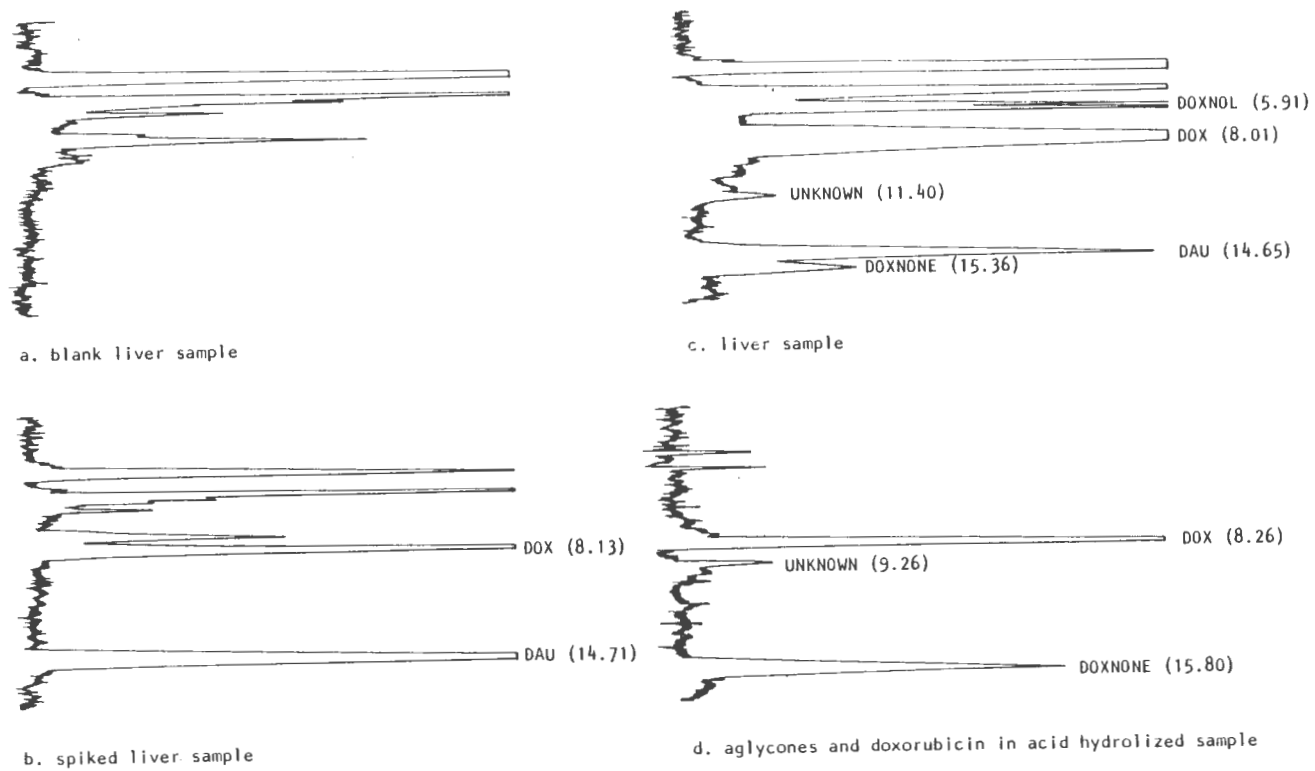


Figure 6. The separation profiles of liver samples in a HPLC system consisted of a C-18 column and a mobile phase of 0.01 M phosphoric acid in 30 % acetonitrile with a flow rate of 0.8 ml/min

a.

chromatographic parameter	DOX	DOXNOL	DOXNCNE	DAU
retention time (min)	8.01	5.91	15.36	14.65
peak width (min)	2.0	0.8	1.3	1.4
capacity factor (k')	1.26	0.67	3.34	3.14
effective plates (N)	257	873	2234	1752

b. Selectivity (α)

	DOX	DOXNOL	DOXNCNE	DAU
DOX	1.00	1.88	2.65	2.49
DOXNOL	1.88	1.00	4.98	4.69
DOXNCNE	2.65	4.98	1.00	1.06
DAU	2.49	4.69	1.06	1.00

c. Resolution (R)

	DOX	DOXNOL	DOXNCNE	DAU
DOX	0.00	1.50	4.45	3.90
DOXNOL	1.50	0.00	9.00	7.94
DOXNCNE	4.45	9.00	0.00	0.52
DAU	3.90	7.94	0.52	0.00

DOX: doxorubicin
 DOXNOL: doxorubicinol
 DOXNCNE: doxorubicincne
 DAU: daunorubicin

Table 2. The chromatographic parameters of a HPLC system consisted of a μ Bondapak C-18 column and a mobile phase of 0.01M H_3PO_4 in 30 % acetonitrile solution at a flow rate of 0.8 ml/min.

that retention times of doxorubicinol, doxorubicin and daunorubicin was 3.5, 4.7, and 8.0 minutes respectively in a LiChrosorb RP-8 column and a mobile phase of 0.01 M H_3PO_4 in 31 % acetonitrile (76). Although the column used in this study was a μ Bondapak C-18 column, Eksborg stated that elution pattern was the same as for a RP-8 column and capacity factors were increased by 0.2 to 0.3 units on a log scale. In comparison with Eksborg's data, the position of doxorubicinol peak was certain, the verification of doxorubicinone was performed by coinjection of the hydrolyzed products of doxorubicin.

b. The monoammonium phosphate-methanol system

This HPLC system consisted of the same μ Bondapak C-18 column and a mobile phase of 0.01 M monoammonium phosphate solution and methanol (35:65). The chromatograms obtained are shown in Fig. 7. The same void volume of 2.83 ml was used to calculate the chromatographic parameters (Table 3). Doxorubicinol was free from interferences in this system and resolution between doxorubicinone and daunorubicin was improved. But resolution between doxorubicin and doxorubicinol was poor. A great disadvantage of this system is the low sensitivity, which was only 36.31 % of the most sensitive one but was not too bad compared with the mobile phase that barely separated doxorubicinol from

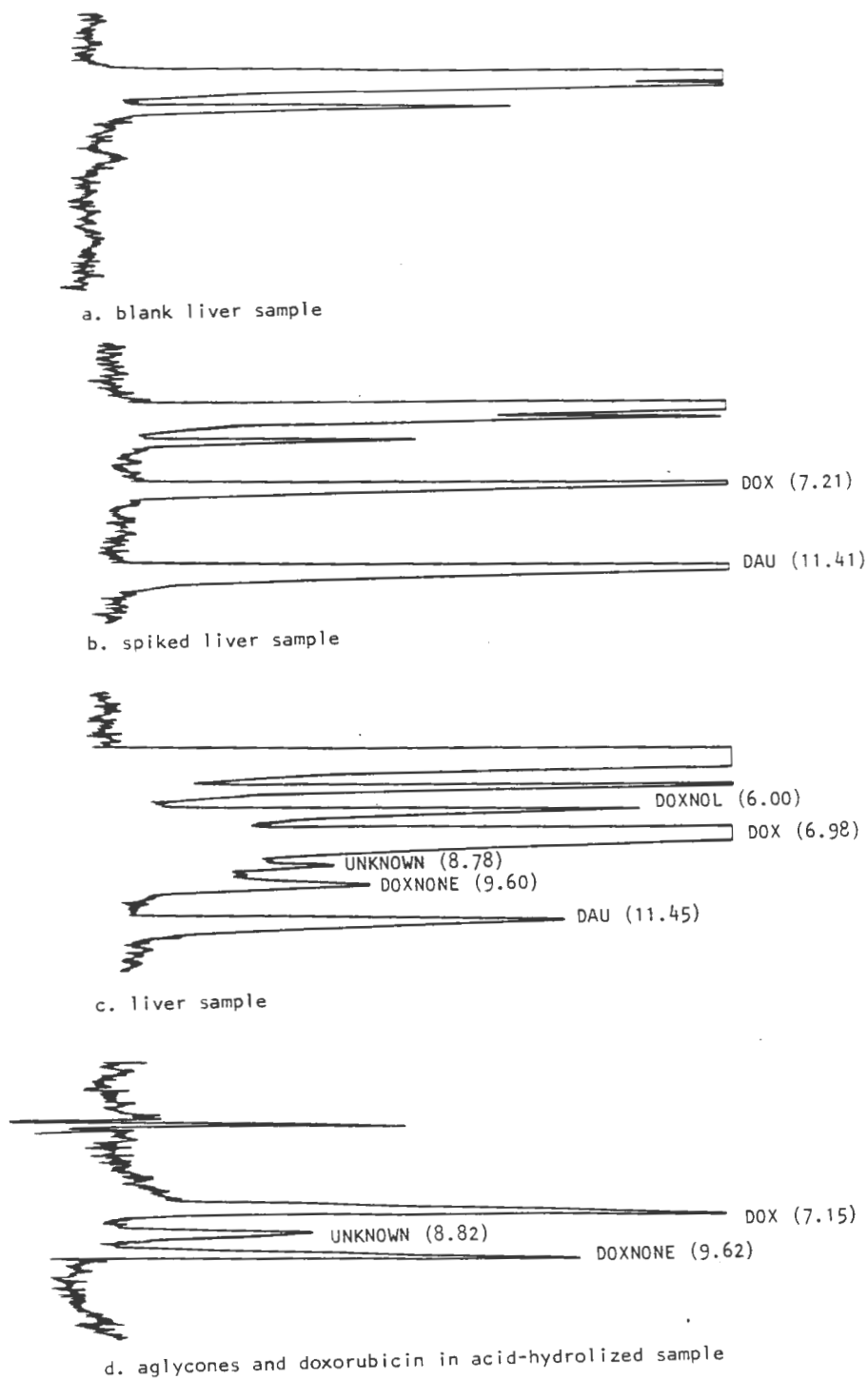


Figure 7. The chromatograms of doxorubicin and related compounds in a HPLC system consisted of a C-18 column and a mobile phase of 0.01 M $\text{NH}_4\text{H}_2\text{PO}_4$ solution and methanol with a flow rate of 1.0 ml/min

a.

chromatographic parameter	DOX	DOXNOL	DOXNCNE	DAU
retention time (min)	6.98	6.00	9.60	11.45
peak width (min)	1.5	0.7	0.7	1
capacity factor (k')	1.47	1.12	2.39	3.04
effective plates (N)	346	1176	3009	2098

b. Selectivity (α)

	DOX	DOXNOL	DOXNCNE	DAU
DOX	1.00	1.31	1.62	2.07
DOXNOL	1.31	1.00	2.13	2.71
DOXNCNE	1.62	2.13	1.00	1.27
DAU	2.07	2.71	1.27	1.00

c. Resolution (R)

	DOX	DOXNOL	DOXNCNE	DAU
DOX	0.00	0.89	2.38	3.57
DOXNOL	0.89	0.00	5.14	6.40
DOXNCNE	2.38	5.14	0.00	2.18
DAU	3.57	6.40	2.18	0.00

DOX: doxorubicin
 DOXNOL: doxorubicinol
 DOXNCNE: doxorubicincne
 DAU: daunorubicin

Table 3. The chromatographic parameters of a HPLC system consisted of a μ Bondapak C-18 column and a mobile phase of 0.01M $\text{NH}_4\text{H}_2\text{PO}_4$ solution and methanol (35:65) at a flow rate of 1 ml/min

the rest in 1.a (53.26 %).

Resolution between doxorubicin and doxorubicinol was improved by lowering the methanol fraction to 0.6, as shown in the chromatograms in Fig. 8. Chromatographic parameters were calculated and showed a general improvement in selectivities and resolutions (Table 4). Simultaneously, another 15 % decrease in fluorescent intensities was sacrificed for this improvement. Due to the great concentration differences between the tissue levels of doxorubicin and doxorubicinol, a good resolution between these two compounds was crucial for the accurate detection of doxorubicinol. As shown in Fig. 8, the very large peak of doxorubicin overlapped the peak of doxorubicinol to some degree in the mobile phase having 65 % MeOH. Therefore, this system of a μ Bondapak C-18 column with mobile phase being a mixture of 0.01 M monom ammonium phosphate solution and methanol (40:60) was the analysis system of choice for all biological samples.

Chromatographic data obtained by Strauss et al. (37) and by Haneke et al. (77) showed the concomitant retention times of doxorubicin, doxorubicinol, doxorubicinone and daunorubicin in this study. A study by Watson and Chan (31) demonstrated the existence of an unknown aglycone and coincided with the peak marked 'unknown' in Fig. 7 and 8,

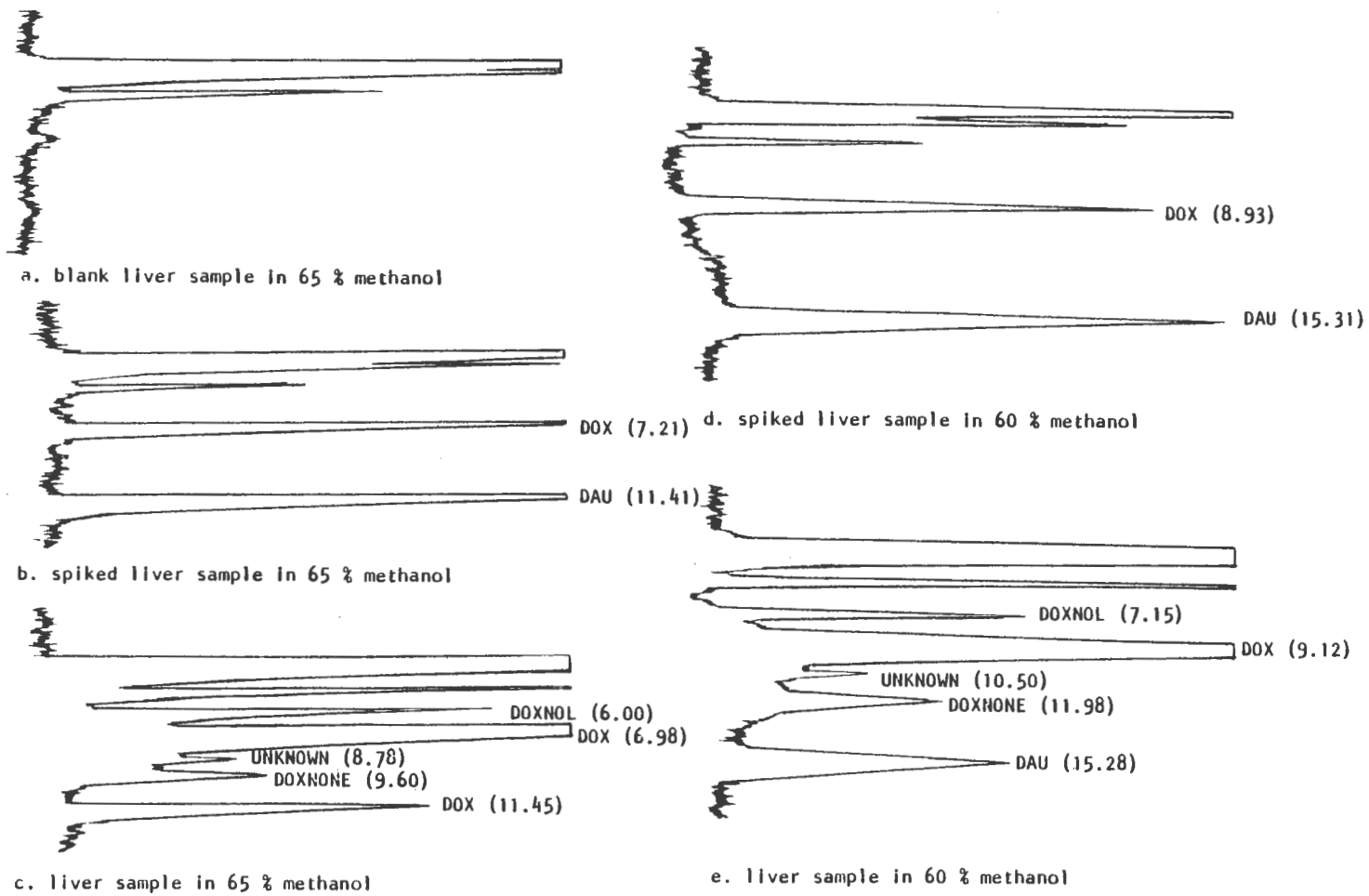


Figure 8. The chromatograms of liver samples in the monoammonium phosphate-methanol system

a.

chromatographic parameter	DOX	DCXNCL	DOXNONE	DAU
retention time (min)	9.12	7.15	11.98	15.28
peak width (min)	1.8	0.6	1.1	1.7
capacity factor (k')	2.22	1.53	3.23	4.40
effective plates (N)	412	2272	1898	1293

b. Selectivity (α)

	DOX	DCXNOL	DCXNCNE	DAU
DOX	1.00	1.45	1.45	1.98
DOXNCL	1.45	1.00	2.11	2.88
DOXNONE	1.45	2.11	1.00	1.36
DAU	1.98	2.88	1.36	1.00

c. Resolution (R)

	DOX	DOXNOL	DOXNCNE	DAU
DOX	0.00	1.64	1.97	3.52
DOXNOL	1.64	0.00	5.68	7.07
DCXNCNE	1.97	5.68	0.00	2.36
DAU	3.52	7.07	2.36	0.00

DOX: doxorubicin
 DCXNCL: doxorubicinol
 DOXNONE: doxorubicinone
 DAU: daunorubicin

Table 4. The chromatographic parameters of a HPLC system consisted of a μ Bondapak C-18 column and a mobile phase of 0.01M $\text{NH}_4\text{H}_2\text{PO}_4$ solution and methanol (40:60) at a flow rate of 1 ml/min

of which the chemical structure has yet to be defined.

c. The normal phase system

This HPLC system consisted of a Radial-pak chromatographic cartridge and a mobile phase prepared from chloroform, methanol, 3.0 mM magnesium chloride solution and glacial acetic acid (72:21:4:3). The void volume was measured to be 1.90 ml by injection of toluene while mobile phase was methylene chloride. The chromatograms of doxorubicin and its metabolites were shown in Fig. 9. Chromatographic parameters were calculated and listed in Table 5.

Among these parameters, the small capacity factors of doxorubicinone and daunorubicin resulted the impossible detection of these compounds in liver samples (Fig. 9). The resolution between doxorubicinone and daunorubicin was also poor. Improvements in mobile phase should be made to overcome these shortcomings. Verification of the peak of doxorubicinol could be done by comparing the data from Baurain et al. (40) and from Chan et al. (46). And verification of doxorubicinone was done by coinjection of the hydrolyzed products of doxorubicin.

However, the inferiority in sensitivity and the

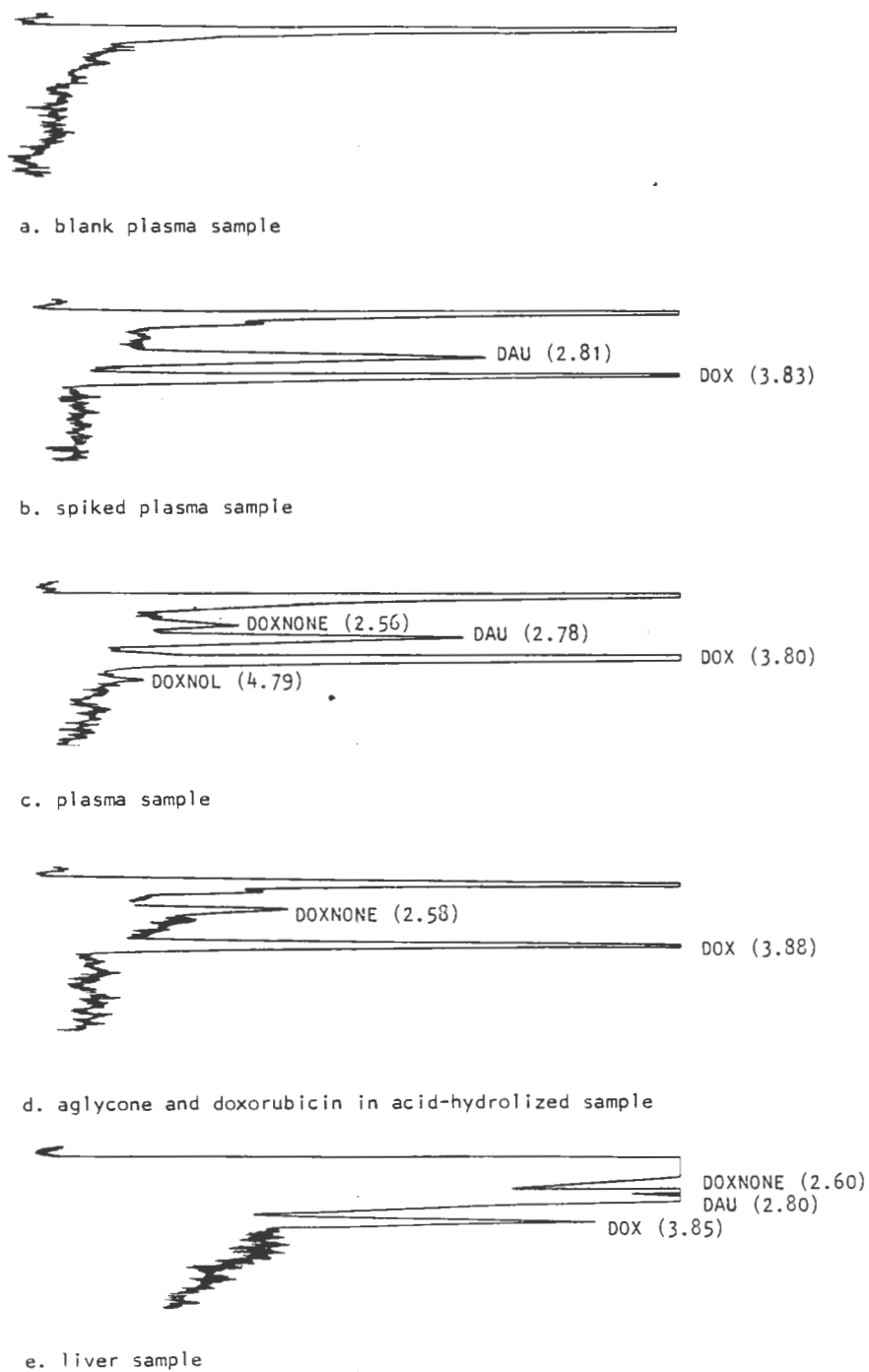


Figure 9. The chromatograms of doxorubicin and related compounds in the normal phase system

a.

chromatographic parameter	DOX	DOXNOL	DOXNCNE	DAU
retention time (min)	3.80	4.79	2.26	2.78
peak width (min)	0.8	0.2	0.5	0.6
capacity factor (k')	3.00	4.04	1.38	1.93
effective plates (N)	361	2294	327	343

b. Selectivity (α)

	DOX	DOXNOL	DOXNCNE	DAU
DOX	1.00	1.35	2.17	1.55
DOXNOL	1.35	1.00	2.93	2.09
DOXNCNE	2.17	2.93	1.00	1.40
DAU	1.55	2.09	1.40	1.00

c. Resolution (R)

	DOX	DOXNOL	DOXNCNE	DAU
DOX	0.00	1.65	2.37	1.46
DOXNOL	1.65	0.00	5.62	4.02
DOXNCNE	2.37	5.62	0.00	0.94
DAU	1.46	4.02	0.94	0.00

DOX: doxorubicin
 DOXNOL: doxorubicinol
 DOXNCNE: doxorubicinone
 DAU: daunorubicin

Table 5. The chromatographic parameters of a normal phase HPLC system of a Radial-pak B column and a mobile phase of chloroform, methanol, 3.0 mM magnesium chloride solution, and glacial acetic acid (72:21:4:3) at flow rate of 2.0 ml/min

employment of the hazardous, expensive chloroform made this system unfavorable. No specific application was suitable for this system. Additionally, this normal phase column required a lengthy time, 30 minutes vs. 10 minutes of the reversed phase column, to equilibrate it. This system, however, did provide another measurement to verify the existence of doxorubicinol. The retention times of doxorubicinol in all three systems, upon comparison with those of the literature, showed similar results. Although standard compound of doxorubicinol was unavailable, it did exist in the tissue samples of rats and was able to be detected by each system but with variable resolution.

2. The Sep-pak Extraction Method

a. Fluorescence of extracted doxorubicin and daunorubicin

The fluorescence of doxorubicin and daunorubicin were measured after extraction from spiked plasma and tissue samples. The retention time of each compound remained consistent throughout the analysis. Calibration graphs were established, therefore, by the peak height ratio of these two compounds versus the spiked concentration of doxorubicin only, without the inclusion of retention time as suggested by Hulhoven and Desager (38). Figs. 10, 11, 12, 13, were thus obtained. All these standard

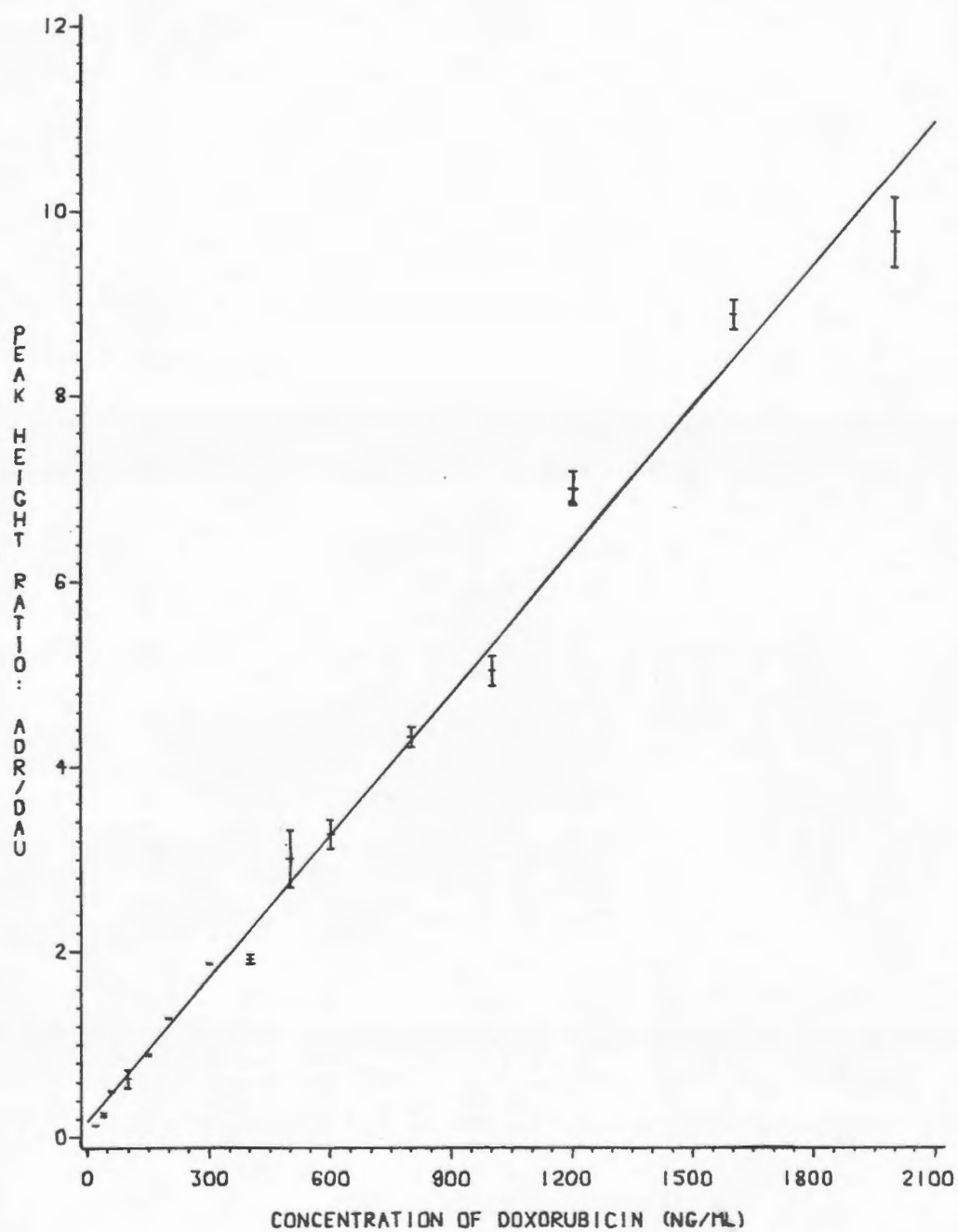


Figure 10. Calibration graph for doxorubicin in human plasma by the organic solvent extraction method

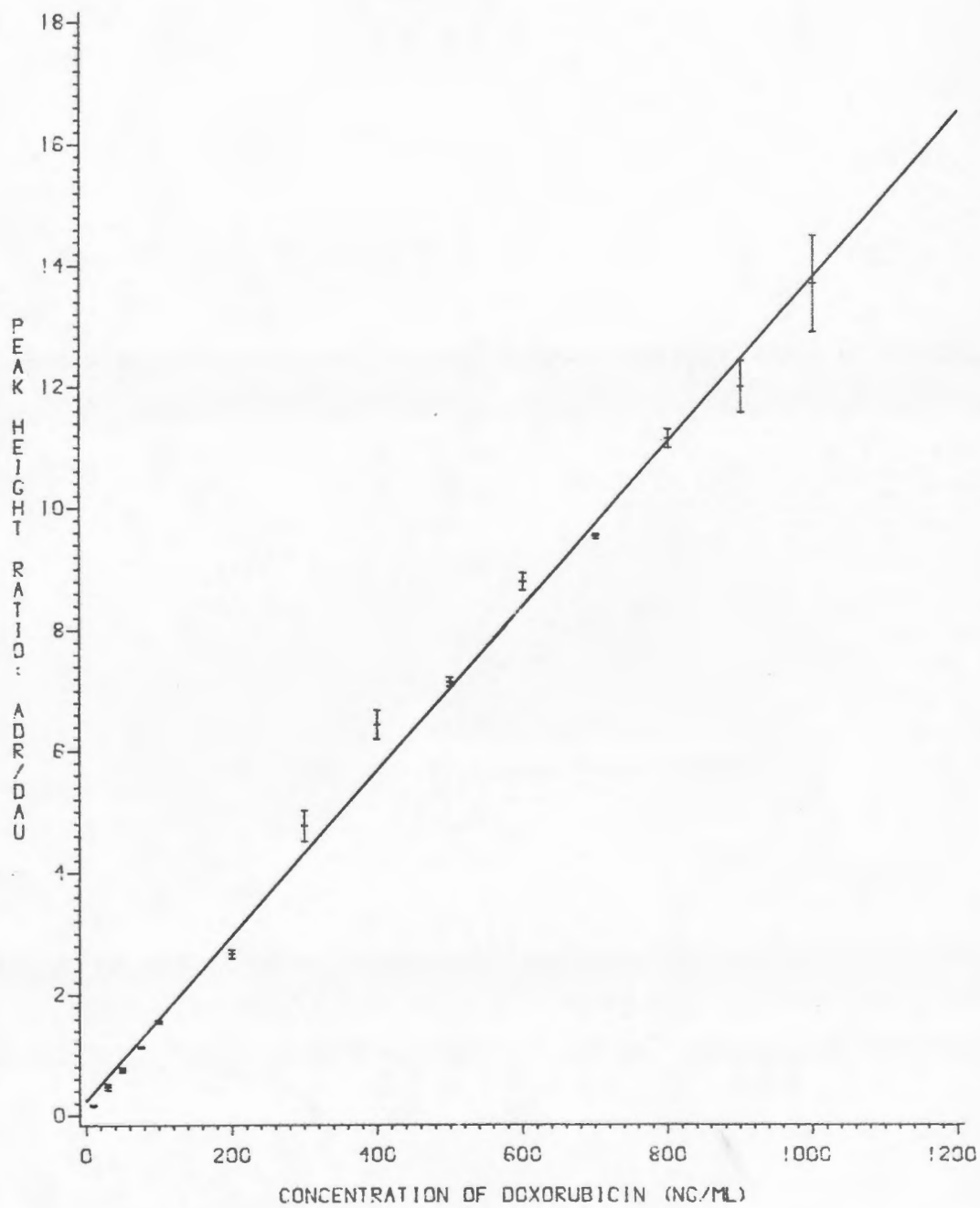


Figure 11. Calibration graph for doxorubicin in human plasma by the Sep-pak method

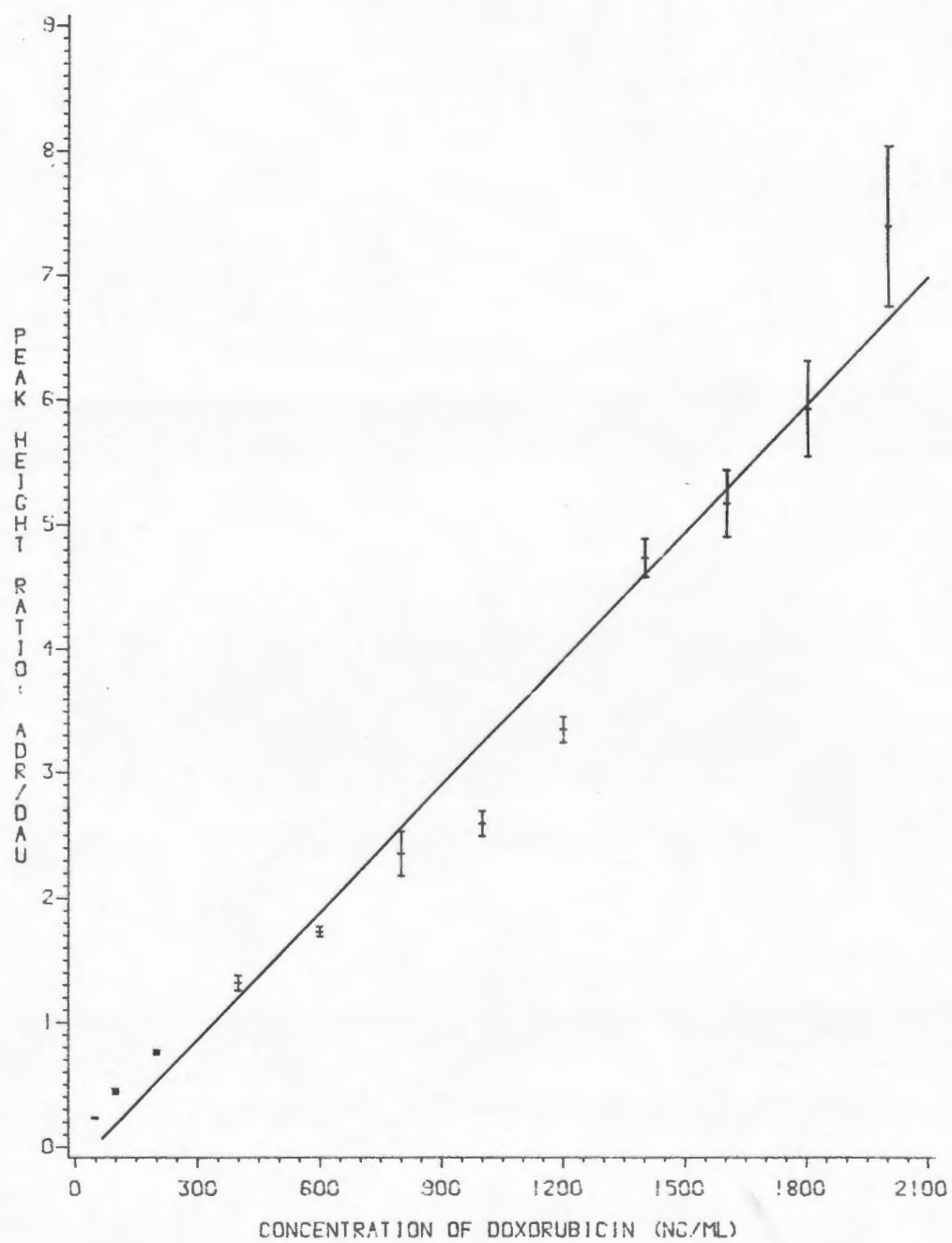


Figure 12. Calibration graph for doxorubicin in rats' liver by the Sep-pak method

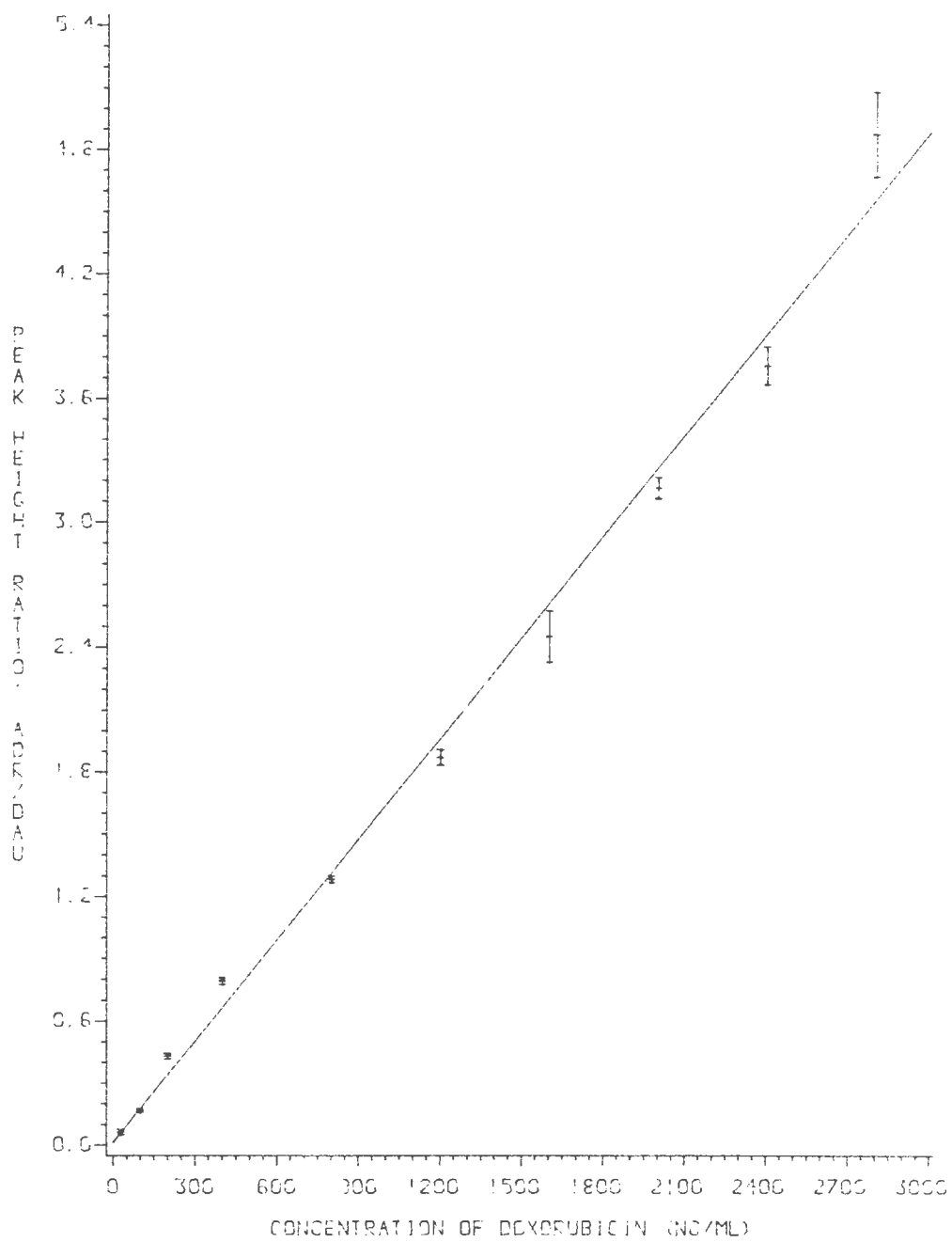


Figure 13. Calibration graph for doxorubicin in rats' kidney by the Sep-pak method

curves showed a good linear relationship (regression coefficients were greater than 0.98) between peak heights ratios and concentrations of doxorubicin.

All sample injection was performed by the automatic sample processor (WISP, Waters) and the quantitative determination was calculated as peak height by the data acquisition system (Data Module, Waters). The coupling of these two instruments and the fluorescent detector was tested for its accuracy and sensitivity. The sensitivity varied with the mobile phase and chromatographic column used in the analysis. The lowest limit of sensitivity for doxorubicin was 5 ng/ml for the chromatographic conditions specified in Figs. 10, 11, and 15 ng/ml for those specified in Figs. 12, 13. The accuracy was determined by three repeated injections of an appropriate sample at 5, 10, 20, 25, 40, 50, 70, 100, 150, 180, 200 μ l respectively and the coefficient of variation was an average of 6.1 ± 0.4 %.

The addition of the internal standard, daunorubicin before extraction or homogenization was to compensate for the differences in matrix and sample volume. In addition, the corresponding changes in daunorubicin concentrations allowed the detection of the wide physiological range of doxorubicin levels (10-1,000 ng/ml) in plasma samples (15, 23, 25, 31, 44, 73-75). A wider concentration difference

was observed between the levels of doxorubicin (the highest could be 40 $\mu\text{g}/\text{Gm}$) and those of its metabolites (the lowest could be 50 ng/Gm) in tissue samples (18,78-80). And the simultaneous detection of these compounds in tissues made the adjustment of daunorubicin concentration unfeasible. A solution was suggested to make separate assays for doxorubicin and its metabolites, each sample with an appropriate weight of tissue. This would be feasible since extraction procedure was not affected by changes in tissue weight in the range between 100 - 400 mg and complete homogenization was assured (Table 6). Besides, this alteration would be advantageous to avoid the self-aggregation phenomenon of doxorubicin, particularly dimerization. Doxorubicin begins to form dimer at 0.5 $\mu\text{g}/\text{ml}$ and this phenomenon can significantly reduce more than 10 % of the fluorescent intensities when the concentrations are more than 5 $\mu\text{g}/\text{ml}$ (47).

Although peak height ratios showed small variation (smaller than 10 %) in the calibration curves, these were performed with only one sample per concentration of doxorubicin (Appendix). Variation in peak height ratios was tested again with 6 samples per concentration as shown in Table 7. These results also showed coefficients of variation to be smaller than 10 % and demonstrated that the Sep-pak method was accurate and precise for doxorubicin

tissue weight ^{*1,2,3} (mg)	peak height ratio	coefficient of variation
liver 102.4 _± 5.3	0.5732 _± 0.0104	1.82
liver 372.2 _± 7.1	0.5762 _± 0.0295	5.12
kidney 98.5 _± 4.3	0.6166 _± 0.0025	0.40
kidney 388.2 _± 9.4	0.6034 _± 0.0133	2.20

- *1 Tissue were from one rat of unknown age whose tissues served as blank in this study
- *2 The results of each tissue weight group were obtained by the assays of three spiked tissue samples
- *3 Spiked concentrations of doxorubicin and daunorubicin were 500 ng and 100 ng per sample respectively

Table 6. The relationship between tissue weight and peak height ratio

sample ^{*1,2} (DOX/DAU)	Sep-pak processed plasma sample		standard solution ^{*4}	
	peak height ratio	C.V. ^{*3}	peak height ratio	C.V.
30/200	0.0226±0.0114	5.10	0.2313±0.0114	4.93
100/200	0.6819±0.0064	0.94	0.6792±0.0280	4.14
300/200	1.8870±0.0202	1.07	2.0844±0.1372	6.58
700/800	4.6778±0.0543	1.16	4.7059±0.0850	1.81

*1 samples are expressed as the concentration of doxorubicin (DOX) and daunorubicin (DAU) in ng/ml

*2 The results in each concentration group were obtained by 6 spiked samples

*3 C.V. is the abbreviation of coefficient of variation

*4 Standard solution was referred to DOX and DAU in mobile phase 1

Table 7. Comparison of the variation between Sep-pak processed samples and standard solutions

analysis. Similar results of this test of variation among tissue samples were also observed in Table 6.

b. Recovery rates and silanization

The standard addition method was first used to calculate the recovery rates and a typical result of spiked samples was shown in Table 8. Two unusual phenomena were observed; one was that apparently greater than 100 % of the spiked amount was recovered and the other was that there was a large variation within an individual sample. In order to determine the causes of these phenomena, control groups were assayed and calculated. The results shown in Table 9 demonstrated that even standard solutions of mobile phase and of the acid-methanol mixture suffered these flaws. From this comparison, Sep-pak method was definitely not the cause and, instead, the calculation by the standard addition method was suspected to be the cause. Therefore, the direct method and the elution method were used as different approaches to calculate recovery rates (Table 10).

Results in Table 10 showed vivid differences in recovery rates calculated by these three methods. With the silanized glassware, the recovery rates calculated by the standard addition method, the direct method and the elution

sample* (DOX/DAU)	peak height ratio	C. V. of ratio	Recovery rates	C. V. of recovery
30/800	0.3798 ±0.0339	8.94	122.8 ±13.4	10.91
50/800	0.6426 ±0.0202	3.14	166.4 16.6	13.60
70/800	0.8919 ±0.0829	9.29	122.3 ±8.2	6.72
200/800	1.5885 ±0.1079	6.79	106.1 ±21.4	20.17
400/800	3.2367 ±0.1574	4.86	122.1 ±63.6	52.04
600/800	5.9501 ±0.1034	1.74	114.3 ±6.0	5.30
800/800	6.4997 ±0.0283	0.44	182.6 ±0.9	0.50
1000/800	8.7547 ±0.2728	3.12	185.9 ±34.65	18.64
1200/800	9.9112 ±1.1900	1.92	166.01 ±47.6	28.66
1400/800	12.6124 ±0.2991	2.37	135.6 ±6.8	4.98
1600/800	13.9111 ±0.7718	5.55	271.1 ±285.4	105.27
2000/800	15.5264 ±0.7048	4.54	98.5 ±8.51	8.65

* The results in each sample group were obtained from one sample by three determinations

Table 8. The peak height ratios and recovery rates of spiked kidney samples

concentration* ¹ of doxorubicin (ng/ml)	type of sample		
	mobile phase* ²	acid-methanol mixture* ³	Sep-pak processed solution* ³
30	130.4± 9.3	127.8± 8.3	130.6±18.8
100	151.2±10.4	122.7±27.7	117.1±50.8
300	97.8± 2.8	135.8± 3.3	111.9±14.9
700	130.9±53.9	110.2±15.3	94.2±33.7

*1 The result in each sample group was obtained from three spiked samples

*2 Mobile phase is consisted of 0.01 M phosphoric acid in 32 % acetonitrile

*3 Acid-methanol mixture is the eluent, 5.0×10^{-3} M phosphoric acid in methanol, used in the Sep-pak method

*4 Sep-pak processed solution is prepared from the addition of standard compounds into the concentrated eluent of blank plasma by the Sep-pak method

Table 9. The recovery rates of different types of samples.

sample ^{*1,2}	standard addition		direct method method		elution method
	silanized	non-silanized	silanized	non-silanized	silanized
organic extraction method					
plasma	-	-	88.6 ±10.9	54.8 ±6.4	-
Sep-pak method					
plasma	113.7 ±22.7	83.0 ±14.9	107.2 ±7.0	86.1 ±4.0	102.2 ±5.6
liver	120.5 ±27.7	72.8 ±14.5	188.9 ±6.5	83.0 ±2.1	99.4 ±2.1
kidney	122.1 ±23.2	68.5 ±15.1	158.7 ±6.3	78.0 ±1.3	98.6 ±4.3
lung	129.7 ±27.2	-	201.1 ±5.5	-	96.4 ±7.2
heart	112.6 ±21.4	-	217.2 ±4.8	89.7 ±6.5	92.4 ±5.8
mean ±STD	119.7 ±21.7	74.8 ±17.4	178.6 ±39.3	84.2 ±5.6	97.8 ±5.8
ave.*3 STD	21.9		3.9		5.4

*1 Results in each group were obtained by three spiked samples

*2 Spiked concentrations of doxorubicin and daunorubicin were 500 ng and 1000 ng per sample respectively

*3 Ave.STD represents the averaged standard deviation of each method

Table 10. The recovery rates of spiked biological samples

method were 119.7 ± 21.7 , 178.6 ± 39.3 and 97.8 ± 5.8 % respectively. The results calculated by the standard addition method were not convincing due to the high variation (coefficient of variation averaged 18 %). The high recovery rates (averaged 178.6 %) that calculated by the direct method arose from the high fluorescent blanks in tissue samples. The high variation in recovery rates (39 %) calculated by the direct method was caused by the lower fluorescent background that was observed alone in the plasma samples. The fluorescent absorbances of blank samples of tissues were shown to be twice as high as that of plasma blank. The exclusion of internal standard during extraction (see Methodology) left the high fluorescent background in tissue samples uncorrected and the degree of fluorescent contribution varied.

The elution method, then, seemed to be the only method that reflected the true recovery rates of the extraction procedure. This method was similar to the direct method but did not show a high fluorescent background due to the dilution effect of the large volume of the eluent (7 ml). The slightly lower recovery rates observed in heart and lung samples were probably attributed to the difficulties in homogenizing these tissues. The only disadvantage in using the elution method was the difficulties in determining the extraction efficiency of samples spiked

with low doxorubicin concentration. However, all three methods showed little or no variation when the spiked amount of doxorubicin changed.

The high variation in recovery rates that only observed in the standard addition method were the result of compounded variation in calculation. The peak heights of doxorubicin and daunorubicin were subjected to a coefficient of variation of 6.1 ± 0.4 %. According to the calculation equations used by each method (see Methodology), the standard addition method may have a compounded variation of 36 % while the other two methods have only one single variation. The results in Table 10 agreed very well with the predicted values, i.e., the coefficient of variation was an average of 21.9 % in the standard addition method, 3.9 % in the direct method and 5.4 % in the elution method respectively.

Silanization played an important role during doxorubicin extraction. Table 10 revealed that at least 25% of the doxorubicin was lost to the glass wall and that dimethyldichlorosilane could effectively prevent this adsorption phenomenon.

c. Comparison with other methods

The recovery rates for organic extraction method were lower than those of the Sep-pak method, regardless of the calculation methods used. The Sep-pak method is definitely superior to the organic extraction method in the ease of operation, the required time for processing, and efficiency. Factors such as pH of the aqueous phase, the volume ratio between organic and aqueous phases, the mixing time and separating of phases that should be carefully controlled to obtain maximal efficiency in the organic extraction method were disregarded in the Sep-pak method. The Sep-pak method required only a Sep-pak cartridge and an appropriate solvent for the elution of doxorubicin and related compounds. It also eliminated the consumption of the hazardous chloroform and reduced the processing time from 30 minutes to 1 minute per sample.

The hydrolysis of doxorubicin which may be caused by the strong acidity (pH=2.34) of the acid-methanol mixture could be prevented by adjusting the pH to 4 - 5. The addition of two drops of 20 % NaOH in methanol right after elution from the Sep-pak cartridge were enough to prevent the hydrolytic reaction while 5.2 % of doxorubicin in the control group were hydrolyzed to doxorubicinone. Thus, the hydrolysis of doxorubicin was influenced by pH and needed

incubation time to proceed hydrolysis. The only disadvantage in the Sep-pak method was that Sep-pak cartridge could not be re-used and a reduction of more than 10% in efficiency was observed in the second use. Except this shortcoming, Sep-pak method provides a fast, easy, efficient and convenient way for sample preparation.

Since the extraction efficiency for tissue samples was dependent on a complete extraction from the cellular components, reagents such as silver nitrate, sodium lauryl sulfate and phosphate buffer were investigated for the most efficient conditions. The results of these trials are shown in Table 11. Schwartz proposed the use of 0.1 % sodium lauryl sulfate solution which facilitated the cell lysis and thereby maximize doxorubicin extraction (39). But sodium lauryl sulfate solution failed to be helpful in this study. The pH 7.4 phosphate buffer proposed by Johansen was not efficient (32.4 vs. 83.0 %) when coupled with the Sep-pak method (72). Besides, the phosphate buffer method showed a higher degree of interferences in the blank samples as shown in Fig. 14. Therefore, the use of 50 % silver nitrate solution alone was demonstrated to be the method of choice with respect to both efficiency and resolution.

It should be noted that the difference in peak height

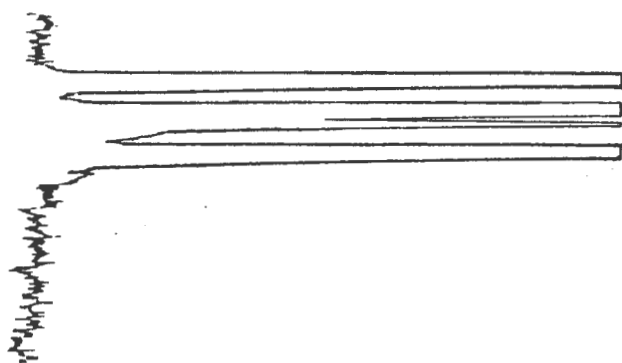
method ^{*1}	peak height ratio	apparent percentage of recovery by the direct method
33% AgNO ₃ 0.1% SLS ^{*2}	1.0368	61.8 ±0.0176±1.6
33% AgNO ₃	1.1020	65.7 ±0.0183±1.1
50% AgNO ₃ 0.1% SLS	1.2875	76.8 ±0.0351±2.1
50% AgNO ₃	1.3917	83.0 ±0.0344±2.1
pH 7.4 ^{*3} buffer	0.5432	32.4 ±0.0219±1.3

*1 This method is the homogenization of 3 spiked liver samples with the reagents indicated and with non-silanized glassware

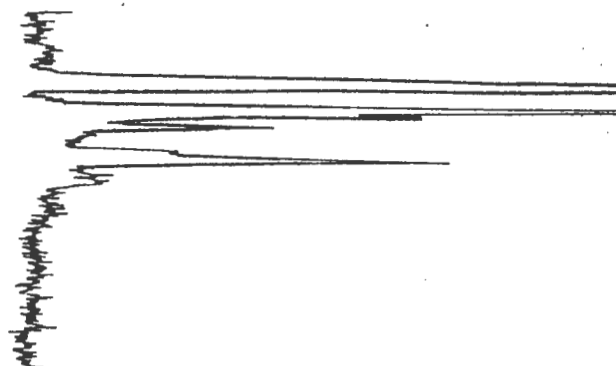
*2 SLS is the abbreviation of sodium lauryl sulfate solution

*3 This buffer is the phosphate buffer of pH 7.4

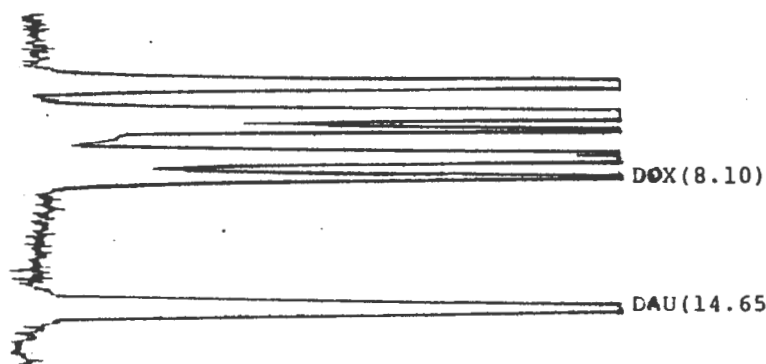
Table 11. The recovery rates of spiked liver samples by different methods



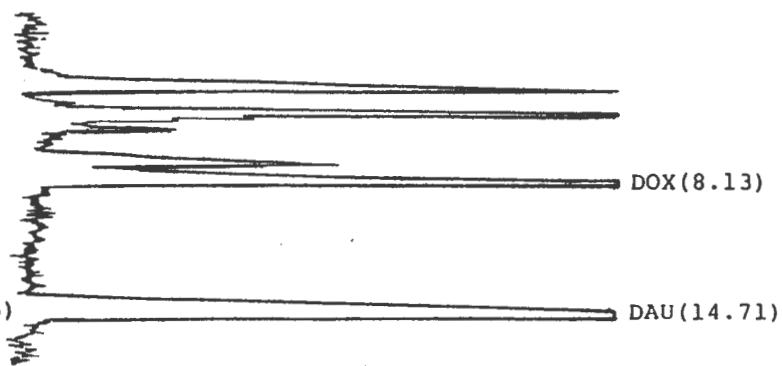
a. blank liver sample by phosphate buffer method



c. blank liver sample by silver nitrate method



b. spiked liver sample by phosphate buffer method



d. spiked liver sample by silver nitrate method

Figure 14. The chromatograms of liver samples processed by the phosphate buffer and the silver nitrate methods. The HPLC system consisted of a C-18 column and a mobile phase of 0.01 M H_3PO_4 in 30 % acetonitrile with a flow rate of 0.8 ml/min.

ratios between adding daunorubicin in the beginning and after the extraction process (homogenization and Sep-pak method) was shown to be almost two-fold. This phenomenon, however, did not exist for plasma samples. The different extraction efficiencies of daunorubicin between plasma and tissue samples indicated that although Sep-pak cartridge had the same efficiency toward both doxorubicin and daunorubicin, almost half of the daunorubicin was not extracted by the homogenization step. This loss of daunorubicin suggested that daunorubicin may not be the internal standard of choice for doxorubicin extraction from tissue, despite the fact that structural difference between these two compounds is only one hydroxyl group. This phenomenon also coincides with the very different pharmacokinetic and pharmacological properties between doxorubicin and daunorubicin.

B. Pharmacokinetic Studies

1. Plasma Samples

Plasma samples from four patients were assayed and the results are shown in Figs. 15,16. Doxorubicin levels in J.C. and M.C. were measured by the organic extraction method while those of the J.M. and A.D. were measured following the Sep-pak approach. Pharmacokinetic parameters, α , β , initial concentration (C_0) and micro-constants such as rate constants of distribution between the first (central) and the second compartments (K_{12} and K_{21}) and rate constant of elimination (K_{EL}), were calculated by AUTCAN. These parameters are listed in Table 12 and the smooth curves in Figs 15,16 represented the simulated plasma levels.

The pharmacokinetic profiles of all four patients' data could be expressed by the two compartment model. The initial concentration (C_0) and the volume of distribution were in a good agreement with the assay methods. These two groups of samples, organic extraction method versus Sep-pak method, were processed separately and these samples arrived at different time. Other than this, the α , β , half-lives and micro-constants (K_{12} , K_{21} and K_{EL}) all showed remarkable agreement. The low regression coefficient (R),

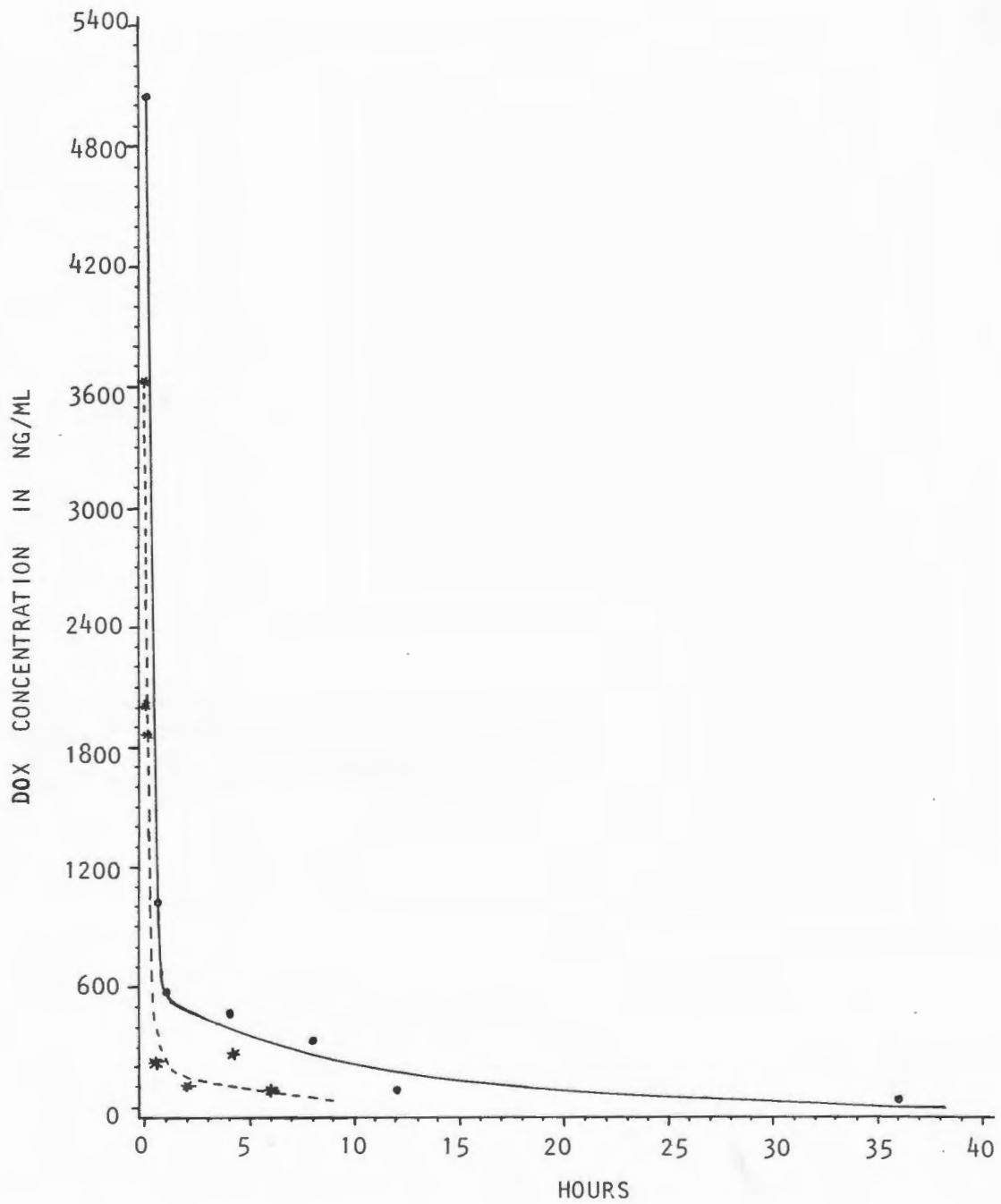


Figure 15. Plasma levels of doxorubicin in two cancer patients; M.C.(., —) and J.C.(*, ---). The smooth curves are the simulated data by a two-compartment model.

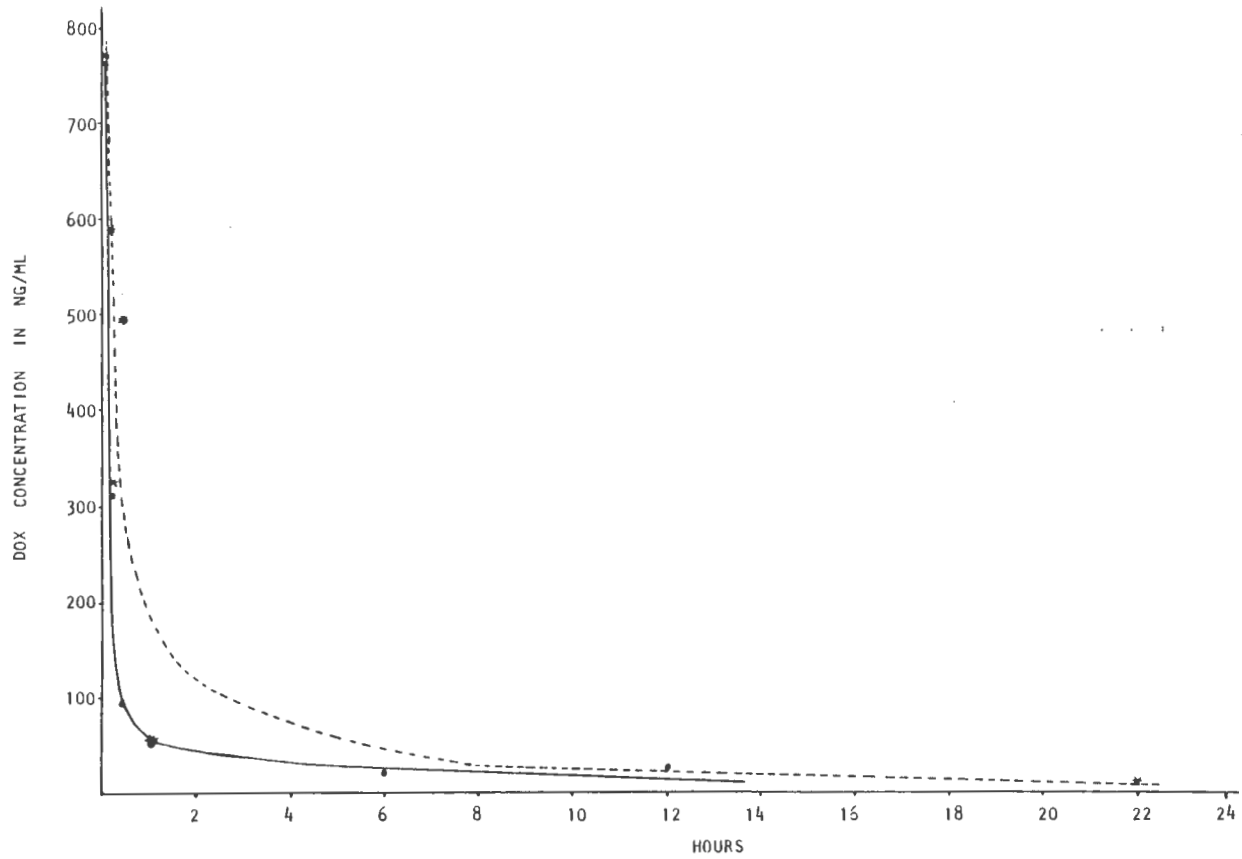


Figure 16. Plasma levels of doxorubicin in two cancer patients; A.D.(., —) and J.M.(*, ---).
The smooth curves are the simulated data by a two-compartment model.

patient	M.D	J.C.	A.D	J.M.
dose (mg)	90	60	64	60
age and sex	F-61	F-72	F-48	M-68
disease status	cervical carcinoma	endometrial carcinoma	colon carcinoma	mesothelial carcinoma
r square	0.9981	0.9505	0.9982	0.8447
alpha (hr^{-1})	5.1506	4.9596	6.0842	4.8380
beta (hr^{-1})	0.0769	0.1210	0.1008	0.1792
$t_{1/2}$ (alpha)	0.134	0.140	0.114	0.143
(hr) (<u>min</u>)	(<u>8.1</u>)	(<u>8.4</u>)	(<u>6.8</u>)	(<u>8.6</u>)
$t_{1/2}$ (beta)	9.01	5.73	6.88	3.83
(hrs)				
C_0 (ng/ml)	7406	4024	1216	943
V_d (liters)	12.15	14.91	52.61	63.61
K_{12} (hr^{-1})	3.8305	4.4985	4.0320	2.0766
K_{21} (hr^{-1})	0.4538	0.3262	0.3168	1.1225
K_{EL} (hr^{-1})	0.8901	1.1362	1.6808	1.0759

Table 12. The pharmacokinetic parameters concerning the plasma samples of four cancer patients

and the large deviation in α , β and micro-constants in J.M. were the results of missing data between 1.0 and 12.0 hours after the administration of doxorubicin. Insufficient volume, less than 0.3 ml, of these samples failed the detection limit of 15 ng/sample specified in the HPLC system and therefore made the quantification impossible.

The initial concentrations of doxorubicin were in agreement with those of the literature, which had C_0 between 1-5 $\mu\text{g/ml}$ (15,44,45,79,80). Only Lee et al. reported initial concentrations after a single bolus i.v. of the usual dosage (40-60 mg/m^2) to be around 10 $\mu\text{g/ml}$ (81). No data from the literature concerning the volume of distribution were available, due to the reported total dosages, for comparison. The half-lives calculated from and also showed similar values as those of the literature (15,23,25,31,44,73-75). The calculated value of the half-life of α phase was 8.0 versus 10 minutes of the literature and that of the half-life of β phase was 6.4 hours in this study versus 10 hours in the literature. The large variation in volume of distribution, however, was unaccountable. From Table 12, it was observed that the youngest patient (A.D.) did exhibit faster rates for both distribution and elimination. Its significance was hard to determine since no other young patients were included in this study. The sex and disease status did not have

significant effects in this study. Metabolites were not detected in any of these four patients.

Data from rat plasma were also analyzed by AUTICAN and depicted in Fig. 17. to show both the observed and the predicted values. Pharmacokinetic parameters were also calculated and listed in Table 13. Due to the missing information of total doses, the volumes of distribution were unable to calculate and thereby missing from this table.

Pharmacokinetic parameters such as α , β , half-lives, C_0 and micro-constants did show a higher degree of variation than those of human plasma samples. Statistically, only group B was different from the rest. A closer observation revealed that the aged groups (C and D) showed higher rate constants of elimination and higher initial concentrations. This observation did not correlate with the physiological conditions of the aged, which usually show a slow distribution phase that leads to higher initial concentrations and a slow elimination phase that leads to lower elimination rates. However, t-test was not performed due to the insufficient samples in each group (n=2).

The difficulties in interpreting the effects of age

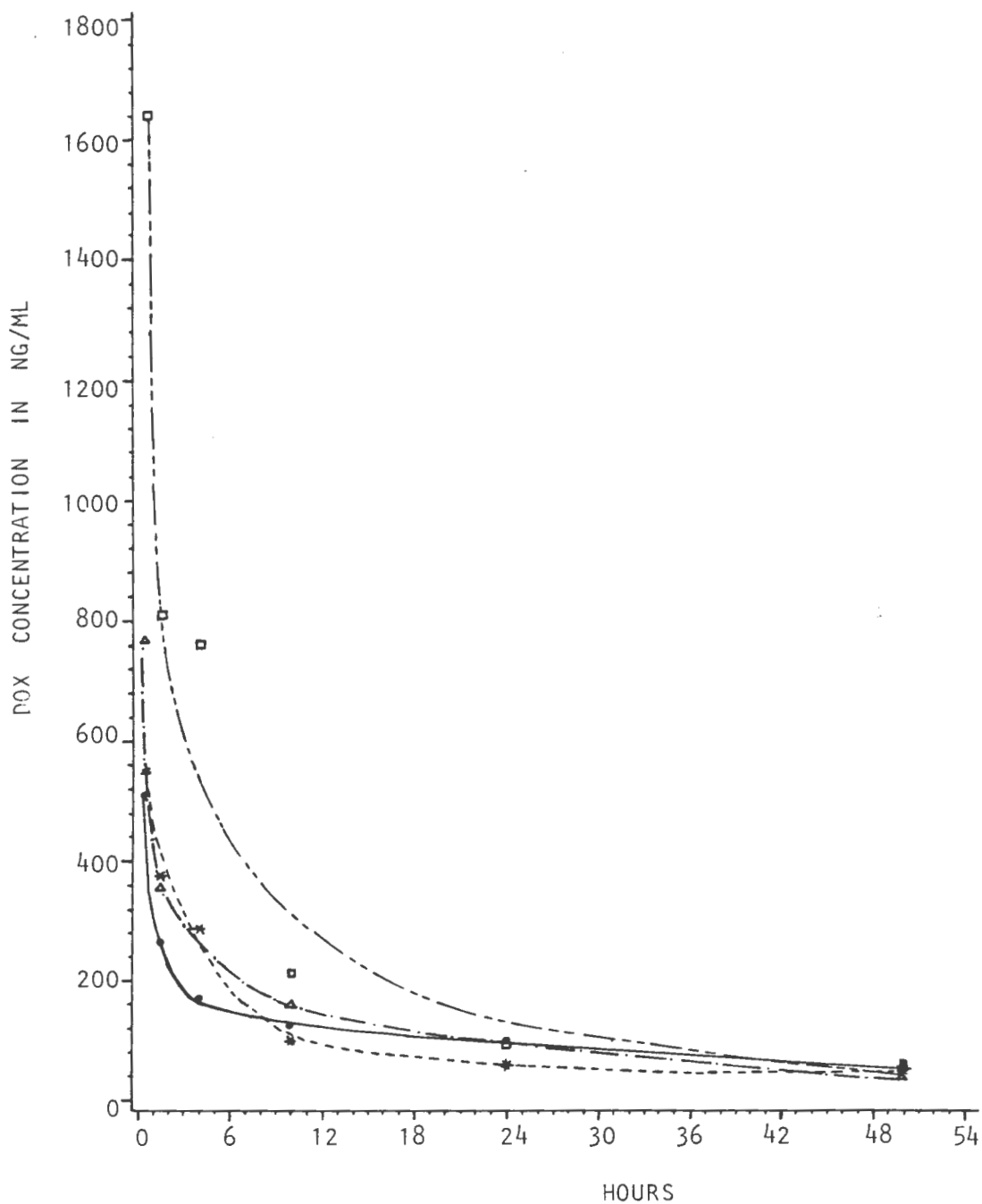


Figure 17. Plasma levels of doxorubicin in rats; the young groups were A(., —) and B(*, ---), and the old groups were C(□, —·—) and D(△, —). The smooth curves are the simulated data by a two-compartment model.

group	A	B	C	D
age	2 months	2 months	2 years	2 years
r square	0.9987	0.775	0.9326	1.000
alpha (hr ⁻¹)	1.2374	0.0062	0.2476	0.0319
beta (hr ⁻¹)	0.0220	0.2800	1.2060	1.3392
t _{1/2} (alpha) (hrs)	0.56	2.48	0.57	0.52
t _{1/2} (beta) (hrs)	31.51	111.80	14.56	21.75
C ₀ (ng. ml)	798	584	3185	1299
K ₁₂ (hr ⁻¹)	0.8595	0.1804	1.0403	0.9487
K ₂₁ (hr ⁻¹)	0.2825	0.0357	0.4048	0.2552
K _{EL} (hr ⁻¹)	0.0962	0.0417	0.3189	0.1673

Table 13. The pharmacokinetic parameter concerning the plasma samples of rats

on doxorubicin pharmacokinetics was partly due to the small sample size and partly due to the poorly defined age groups. The age groups of 2 months and 2 years seemed to fall, respectively, in the very young and the very old parts of the life span of rats. There were no control groups (of medium age) for this study. Since the very young species have similar physiological abnormalities as those of the very old species, such as decreased protein binding, decreased volume of distribution, a higher fat content, reduced metabolism, etc. (59,69-71). One may not necessarily see much differences between these two age groups.

From Fig. 17, a comparison with the literature data was made. The observed doxorubicin levels in rat plasma were significantly lower than those reported by Marafino et al., Sonneveld et al. and Ozaols et al. (16,79,82) but were in good agreement with those of Jchansen, Broggini et al. and Pacciarini et al. (72,83,84) The plasma samples of rats also exhibited two metabolites; doxorubicinol and doxorubicinone, and their concentrations as doxorubicin equivalents were listed in Table 14. These metabolites appeared in the bloodstream almost simultaneously with doxorubicin itself as shown in Figs. 18-21. This phenomenon was also observed in the plasma samples of human and rabbits, which was attributed to metabolism occurred in

group of rats	time* ¹ (hours)	doxorubicin concentration (ng/ml)	doxorubicinol concentration (ng/ml)* ²	doxorubicinone concentration (ng/ml)* ²
A	0.5	512.2± 20.4	492.1±70.9	202.0±47.4
	1.5	267.2± 21.0	339.5±46.2	163.2±12.0
	4.0	171.3± 9.4	183.0±19.6	0.0
	10.0	128.6± 5.2	87.5±17.9	35.7± 3.0
	24.0	103.2± 18.2	80.7± 1.8	101.7±22.7
	50.0	58.6± 11.2	122.8±25.8	0.0
B	0.5	548.6± 25.2	151.2±18.6	0.0
	1.5	373.6± 12.2	131.2±20.1	0.0
	4.0	287.7± 56.5	103.7±19.6	0.0
	10.0	99.2± 11.0	64.2±14.7	0.0
	24.0	62.6± 9.7	21.6± 5.2	0.0
	50.0	53.3± 12.5	53.3± 1.7	0.0
C	0.5	1641.7± 28.5	123.9±12.1	0.0
	1.5	811.1± 42.8	69.0±15.8	0.0
	4.0	763.6±103.8	104.6±17.2	0.0
	10.0	213.6± 21.3	41.8± 6.1	0.0
	24.0	96.2± 0.2	53.5±12.7	0.0
	50.0	60.7±13.6	0.0	0.0
D	0.5	770.2± 6.8	0.0	0.0
	1.5	356.2± 6.7	78.5±15.8	0.0
	10.0	161.4± 21.0	52.0± 4.8	0.0
	50.0	45.1± 8.9	0.0	0.0

*1 The results shown at each time interval in each group were obtained by repeated assays of three samples from one rat.

*2 The concentrations of metabolites (doxorubicinol and doxorubicinone) are expressed as the doxorubicin equivalents.

Table 14. Levels of doxorubicin and its metabolites in rats' plasma by the Sep-pak method

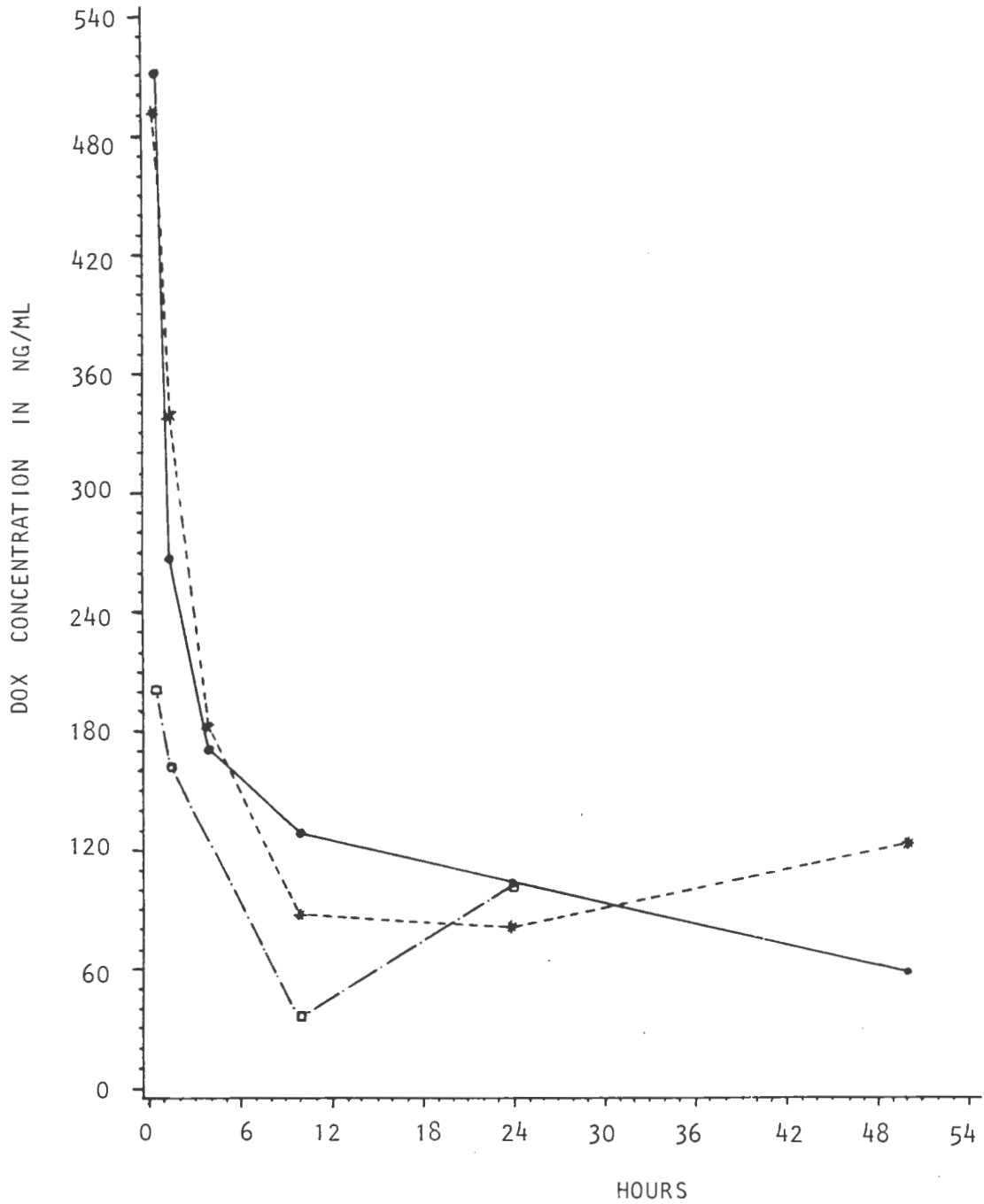


Figure 18. Plasma levels of doxorubicin (., —), doxorubicinol (*, ---) and doxorubicinone (□, —·—) in group A rats. Concentrations of the metabolites are expressed as doxorubicin equivalents.

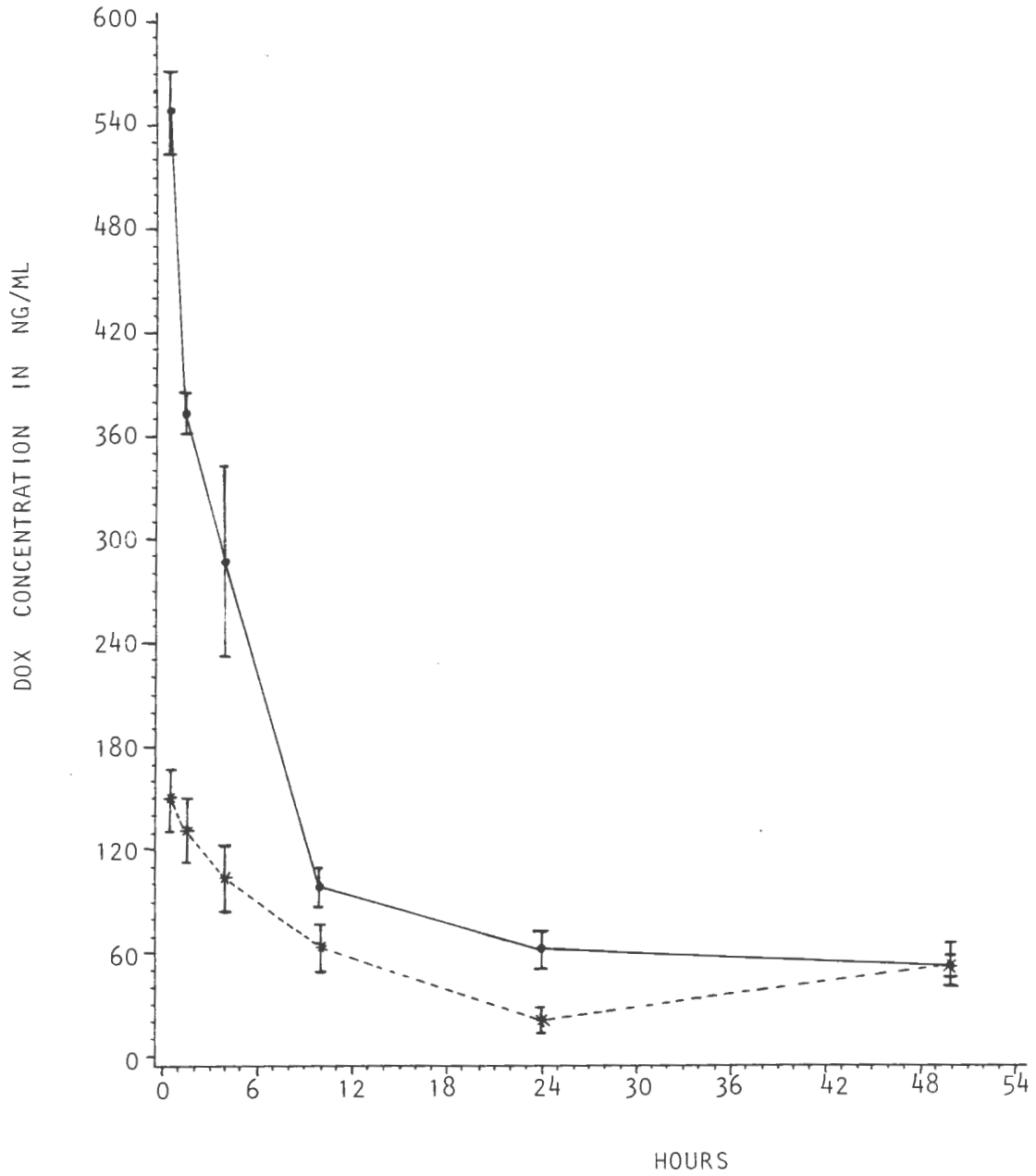


Figure 19. Plasma levels of doxorubicin (., —) and doxorubicinol (*, ---) in group B rats. Concentrations of doxorubicinol are expressed as doxorubicin equivalents.

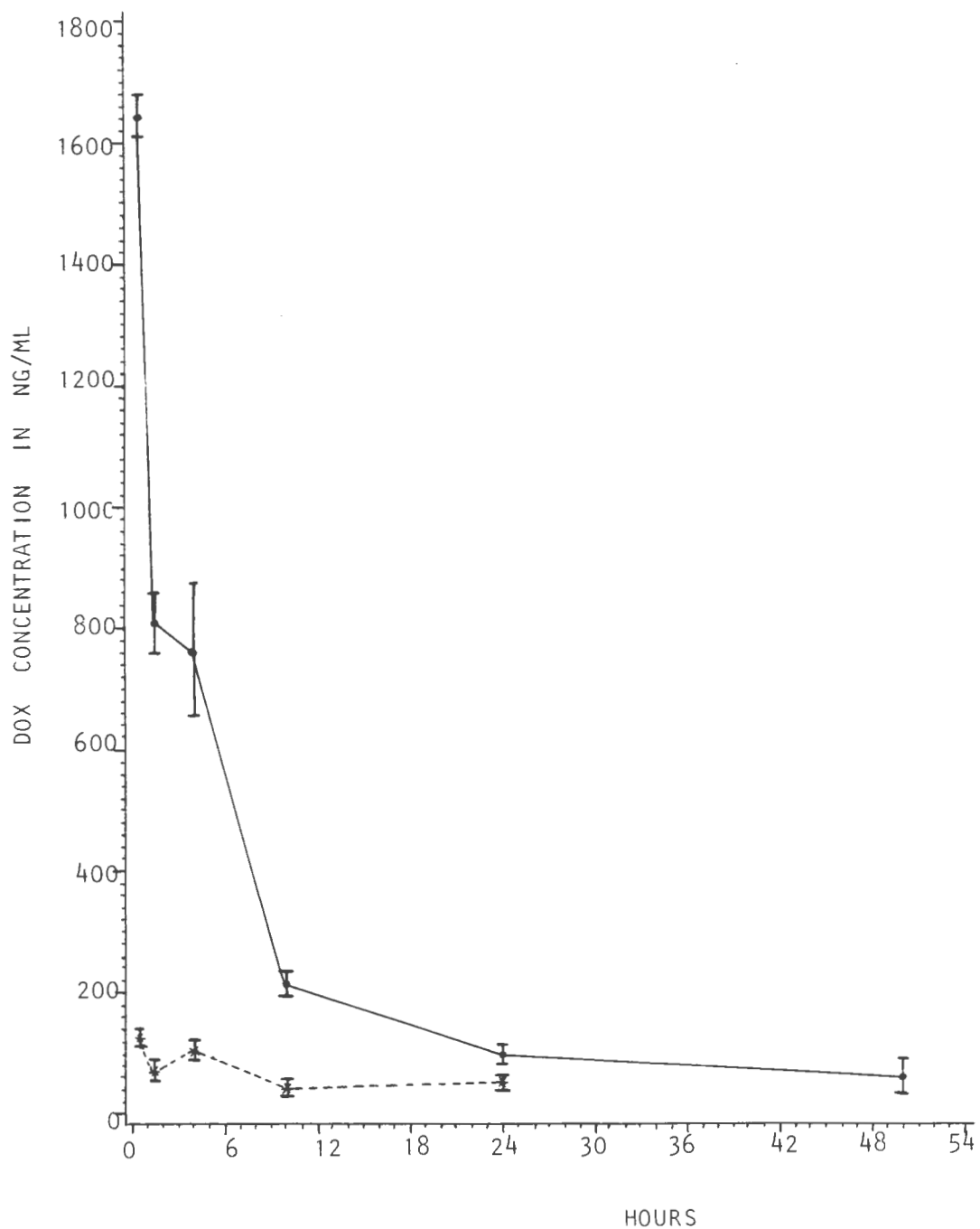


Figure 20. Plasma levels of doxorubicin (., —) and doxorubicinol (*, ---) in group C rats. Concentrations of doxorubicinol are expressed as doxorubicin equivalents.

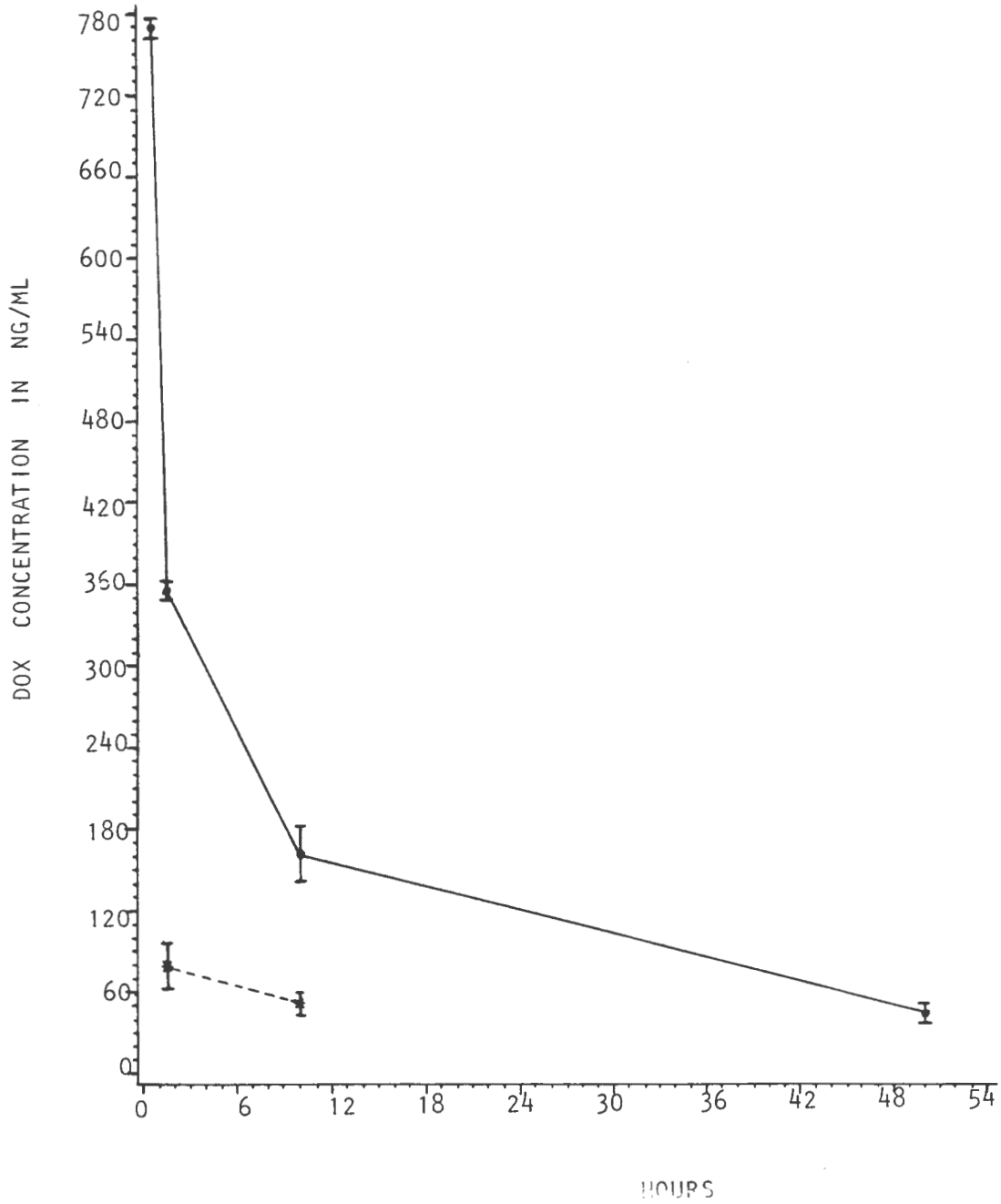


Figure 21. Plasma levels of doxorubicin (., —) and doxorubicinol (*, ---) in group D rats. Concentrations of doxorubicinol are expressed as doxorubicin equivalents

the bloodstream (15,44,45,85). Doxorubicinol levels in rat plasma did not show significant difference between the young and old rats. Only group A was statistically different from the rest. This observation did not correspond with that of doxorubicin levels in which group B was significantly different.

Therefore, this study did not reveal the possible roles of age in doxorubicin pharmacokinetics. The difficulties in analyzing the data from rat plasma arose from the small sample size of each group and the lacking of control groups.

2. Tissue Samples

Kidney samples of four groups of rats were assayed and the results shown in Table 15. A higher variation was observed when concentrations of doxorubicin or doxorubicinol fell below 5 $\mu\text{g}/\text{Gm}$ of tissue. This was due to the detection limit of the HPLC system which, as mentioned before, could not tolerate the signal to noise ratios to fall below 5.

As shown in Fig. 22, there was a 40-fold difference between the concentrations of doxorubicin and doxorubicinol. And the only possible way for accurate

group of rats	time* ¹ (hrs)	DOX* ² conc. (ng/Gm)	C.V.* ³	DOXNOL* ⁴ conc. (ng/Gm)	C.V.
A	0.5	3.57±0.66	18.37	0.0	-
	1.5	42.44±6.67	15.71	1.13±0.27	23.77
	4.0	15.20±1.60	10.54	0.29±0.07	25.38
	10.0	14.11±0.70	4.96	0.25±0.03	13.60
	24.0	9.89±0.98	9.91	0.16±0.01	8.63
	50.0	14.58±1.39	9.56	0.47±0.03	6.56
B	0.5	16.89±0.34	2.04	0.0	-
	1.5	17.27±0.74	4.28	0.15±0.04	23.37
	4.0	18.50±0.19	1.03	0.12±0.03	22.15
	10.0	16.48±1.47	8.91	0.24±0.05	19.94
	24.00	16.94±1.24	7.30	0.52±0.09	17.10
	50.0	7.74±0.66	8.53	0.34±0.05	15.88
C	0.5	28.84±1.79	6.21	0.64±0.11	17.66
	1.5	17.33±1.20	6.92	0.95±0.13	13.60
	4.0	21.48±2.44	11.34	0.80±0.10	12.10
	10.0	34.44±6.55	19.03	0.78±0.14	18.53
	24.0	25.46±5.26	20.66	0.79±0.15	19.47
	50.0	16.07±0.37	2.29	0.89±0.12	13.54
D	0.5	20.66±4.22	20.43	0.08±0.01	14.40
	1.5	36.87±6.46	17.52	0.85±0.14	15.90
	10.0	17.83±0.26	1.47	0.46±0.07	15.54
	50.0	12.02±0.52	4.31	0.43±0.10	22.64

*1 The results shown at each time interval in each group were obtained from repeated assayed of three samples from one rat.

*2 DOX conc. = doxorubicin concentration

*3 C.V. = coefficient of variation

*4 DOXNOL conc. = doxorubicinol concentration expressed as doxorubicin equivalent

Table 15. The levels of doxorubicin and doxorubicinol in rats' kidney

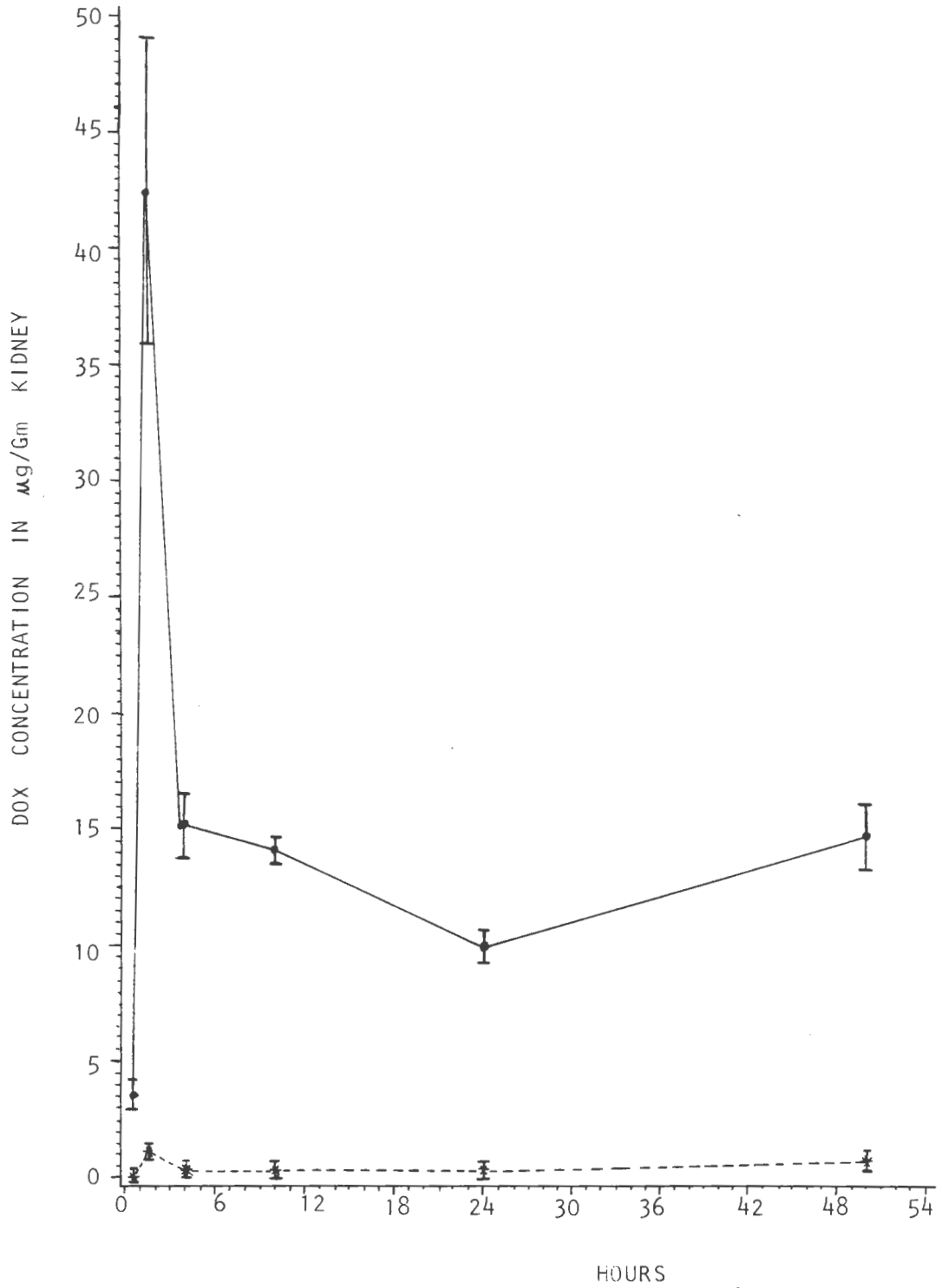


Figure 22. Kidney levels of doxorubicin (., —) and doxorubicinol (*, ---) in group A rats. Concentrations of doxorubicinol are expressed as doxorubicin equivalents.

quantification of both compounds was the separate analyses, as suggested before. Many articles ignored the metabolites in rat tissue samples claiming their amounts were insignificant (18,54,72,79) while other reported high fractions (upto 40 %) of the total doxorubicin equivalents in tissue were the metabolites (78,86). Although the observed results shown in Table 15 coincided with the former, further studies in other tissues such as heart, liver, lung, spleen, brain, etc., should be engaged to confirm the common existence of this phenomenon. If this holds true for all tissue, it would not be worthwhile to carry out another analysis to quantitate such a minor component.

The kidney levels of doxorubicin were only half of those reported in the literature (16,72,83). This was due to inaccurate determination of internal standard whose signal to noise ratio was lower than 5. This was unavoidable since only one concentration of daunorubicin solution was used. However, these errors occurred only for a few samples when doxorubicin concentrations exceeded 20 $\mu\text{g}/\text{Gm}$ of tissue. Since the quantities of kidney metabolites were determined as minor components, future assays of tissue samples should be carried out with a smaller sample size and an appropriate concentration of daunorubicin to cover the range of 8-40 $\mu\text{g}/\text{Gm}$ of tissue.

The smaller sample size of tissue is also encouraged by the fact that the self-aggregation, if happens above 5 $\mu\text{g}/\text{ml}$, will significantly reduce the fluorescent intensity of doxorubicin (47).

A graph of doxorubicin levels in all four groups of rats revealed the same difficulties in recognizing the age effects (Fig. 23). As discussed previously, the small sample size and the lacking of control groups were the causes.

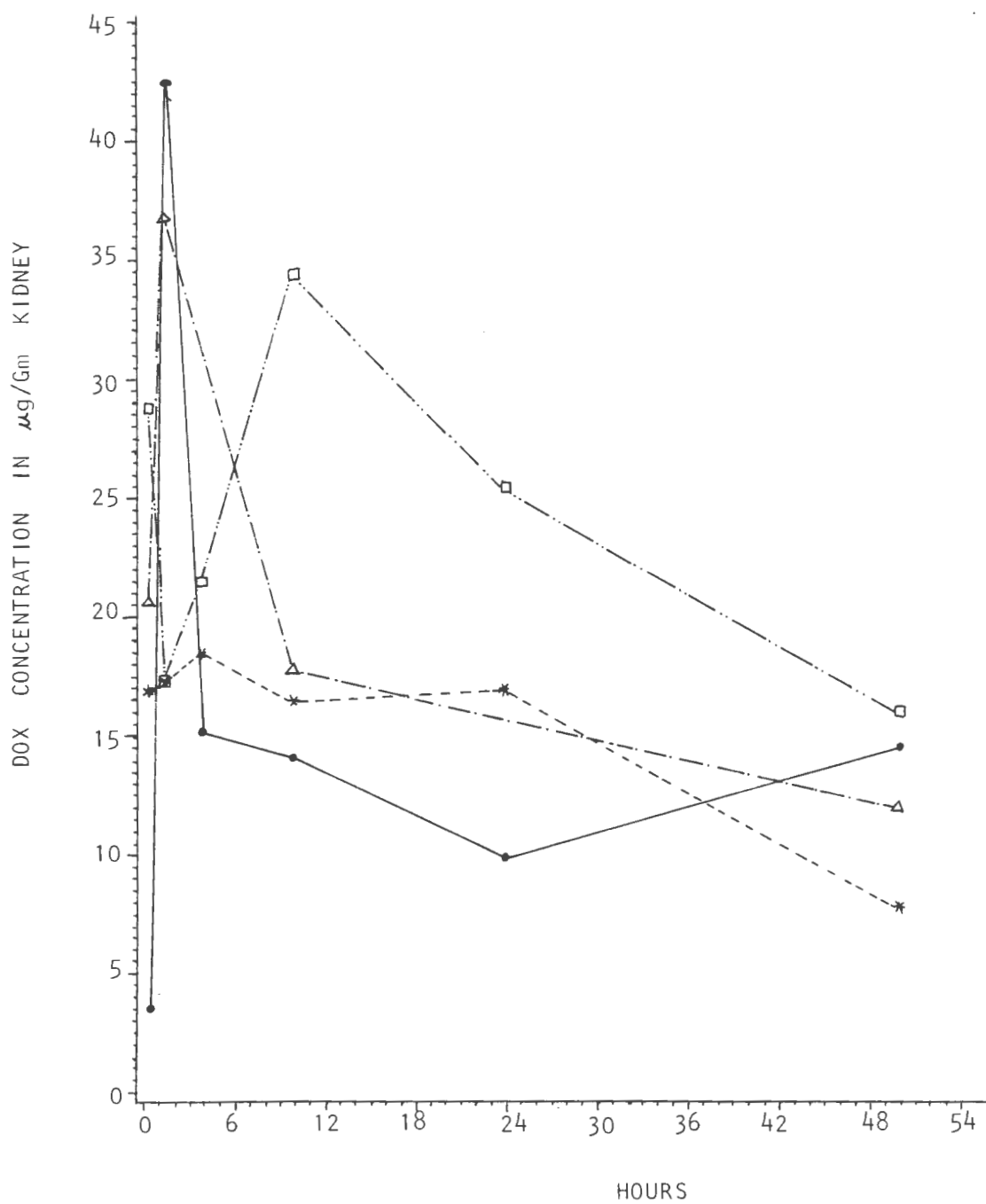


Figure 23. Kidney levels of doxorubicin in rats. Groups A (., —) and B (*, ---) were the young rats, and groups C (□, -·-·) and D (△, —) were the old rats.

C. The Stability Study

1. The Stability of Doxorubicin in Solution

Rates of doxorubicin degradation at various concentrations were first quantified and they were shown to be the same. Therefore, a first order degradation process was assumed. A semi-log plotting procedure was applied to these data and the superimposed straight lines confirmed that the degradation process of doxorubicin was a first order reaction. The stability evaluation was then performed with buffers of various pHs, different buffering systems, water, acid-methanol mixture and mobile phase of the analysis system (0.01 M monoammonium phosphate solution and methanol (40:60)). These rate constants as well as the T_{90} 's, expressed in either hours or days, were listed in Table 16.

The T_{90} would be a more suitable parameter to evaluate the degradation than the conventional T_{50} . Because it indicated the time when only 10 % of doxorubicin in solution has degraded rather than 50 %. This criterion is the maximal time period for doxorubicin solution to exhibit acceptable stability since this antineoplastic agent has such a small therapeutic index (87).

medium	rate constant (hr ⁻¹)	T ₉₀	
		hours	days
PCB ^{#1} pH 2.00	2.16X10 ⁻⁴	487.8	20.3
PCB pH 3.00	1.11X10 ⁻⁴	494.2	39.5
PCB pH 4.00	1.28X10 ⁻⁴	823.1	34.3
PCB pH 5.00	2.00X10 ⁻⁴	526.8	22.0
PCB pH 6.00	3.76X10 ⁻⁴	280.2	11.7
PCB pH 7.00	5.63X10 ⁻⁴	187.1	7.8
PCB pH 8.00	1.41X10 ⁻³	74.7	3.1
PCB pH 10.00	2.38X10 ⁻²	4.43	-
PCB pH 11.00	5.65X10 ⁻²	1.86	-
PCB pH 11.90	0.129	0.82	-
P-B ^{#2} pH 7.40	1.85X10 ⁻²	5.70	-
P-B pH 7.00	1.18X10 ⁻²	8.93	-
A-M ^{#3} pH 2.34	8.97X10 ⁻⁵	1174.8	48.95
H ₂ O pH 5.62	1.82X10 ⁻⁴	585.3	24.39
M.P. ^{#4} pH 4.00	8.32X10 ⁻⁵	1266.6	52.77

*1 PCB is the buffering system consisted of phosphate, citrate and borate

*2 P-B is the phosphate buffer

*3 A-M is the acid methanol mixture used in Sep-pak extraction method

*4 M.P. is the mobile phase used in the HPLC analysis system

Table 16. The rate constants of doxorubicin degradation in various media

From Table 16, it is observed that a change not only in pH but also in the buffering agents showed a different rate of degradation. However, pH did not have a significant effect on degradation once it dropped below 5.00. The variation in these rate constants ($\text{pH} \leq 5.00$) was thought to be a deviation from a true value rather than the effects of pH.

A graph of the rate constants in the same buffer system (the citrate - phosphate - borate buffers) explicitly demonstrated the effect of pH on doxorubicin stability (Fig. 24). This graph showed that the rate constants dropped sharply around pH 11 and doxorubicin was stable in a pH range of 2.0 to 7.0. This phenomenon justified the raising pH of eluents to only 5-6 in the Sep-pak method and the neutralization of ultrafiltrates in the protein binding study.

The buffering agents also demonstrated a significant effect on doxorubicin degradation. The rate constants of solutions of the same pH 7.00 were 5.36×10^{-4} hr for phosphate-citrate-borate buffer and 1.18×10^{-2} hr for phosphate buffer. Such a wide difference in stability cautioned the choice of storage medium or the infusion fluid when long i.v. infusion (upto 96 hours) was chosen to be the route of administration (88). A graph of the

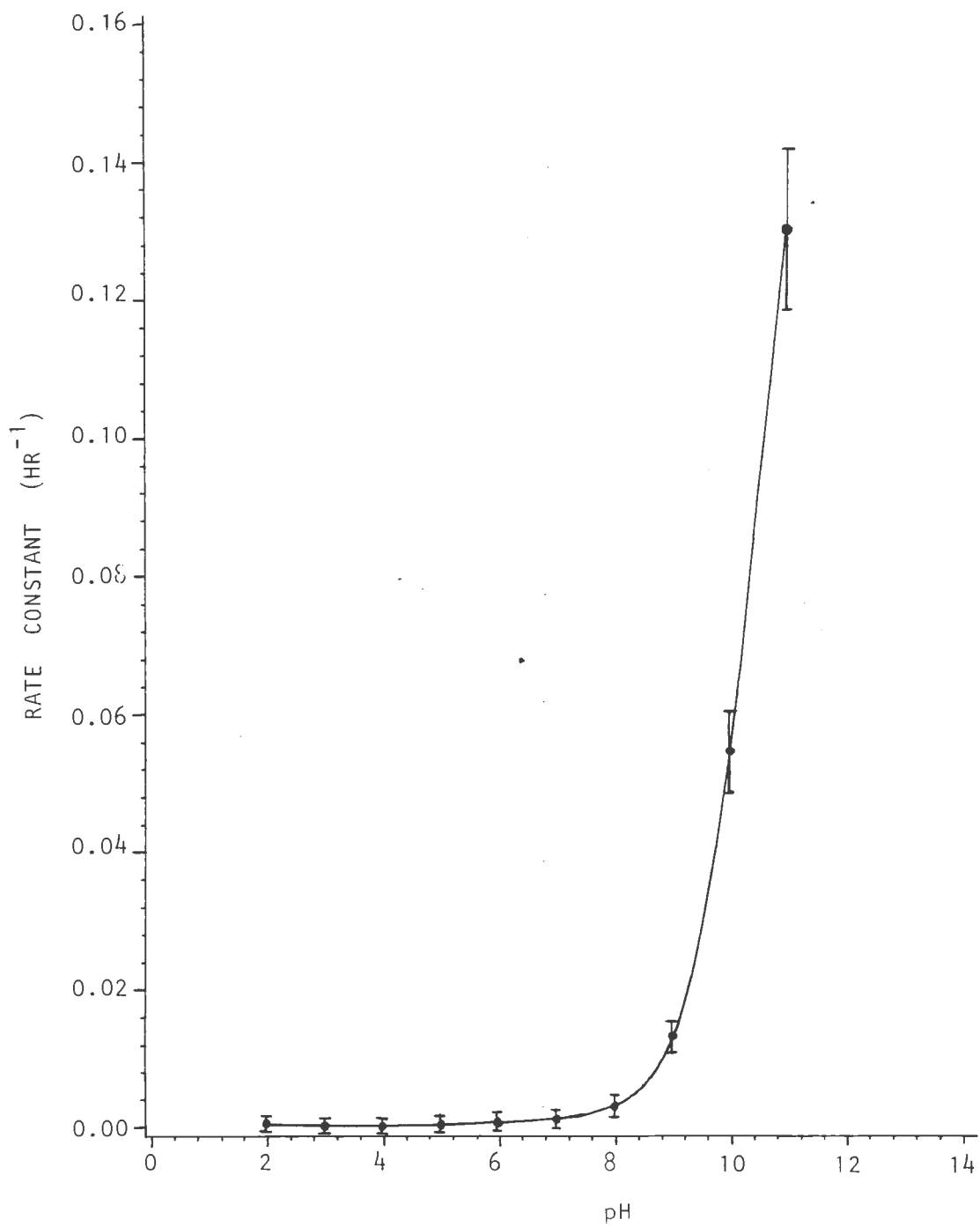


Figure 24. The effect of pH on doxorubicin stability in the citrate-phosphate-borate buffer system

rate constants, expressed as the percentage of doxorubicin remaining, clearly showed the effects of pH and buffering agents (Fig. 25). And the advised storage time of T_{90} could be determined by the points the intercept of an abscissa of 90 %.

The degradation products of doxorubicin hydrochloride have not yet been identified. Since they did not show any chromatographic peaks in any of the three HPLC systems used, they possessed little, if any, fluorescent properties. Therefore, it was impossible to postulate the possible degradation products from doxorubicin ionization in which the ionized species all have fluorescent properties (Fig. 4).

2. The Stability of Doxorubicin in Plasma

The concentration of doxorubicin in spiked frozen plasma samples decreased during storage. This degradation was further aggravated by the number of freezing/thawing cycles (Table 17). Frozen plasma samples, thawed at the intervals of 0, 1, 3, 5 days respectively, were extracted using the Sep-pak method and analyzed chromatographically. While samples experienced a loss of 40 % after 10 freezing/thawing cycles, the control groups lost only 3.9 %, 15.2 %, 29.2 % of the original doxorubicin. An

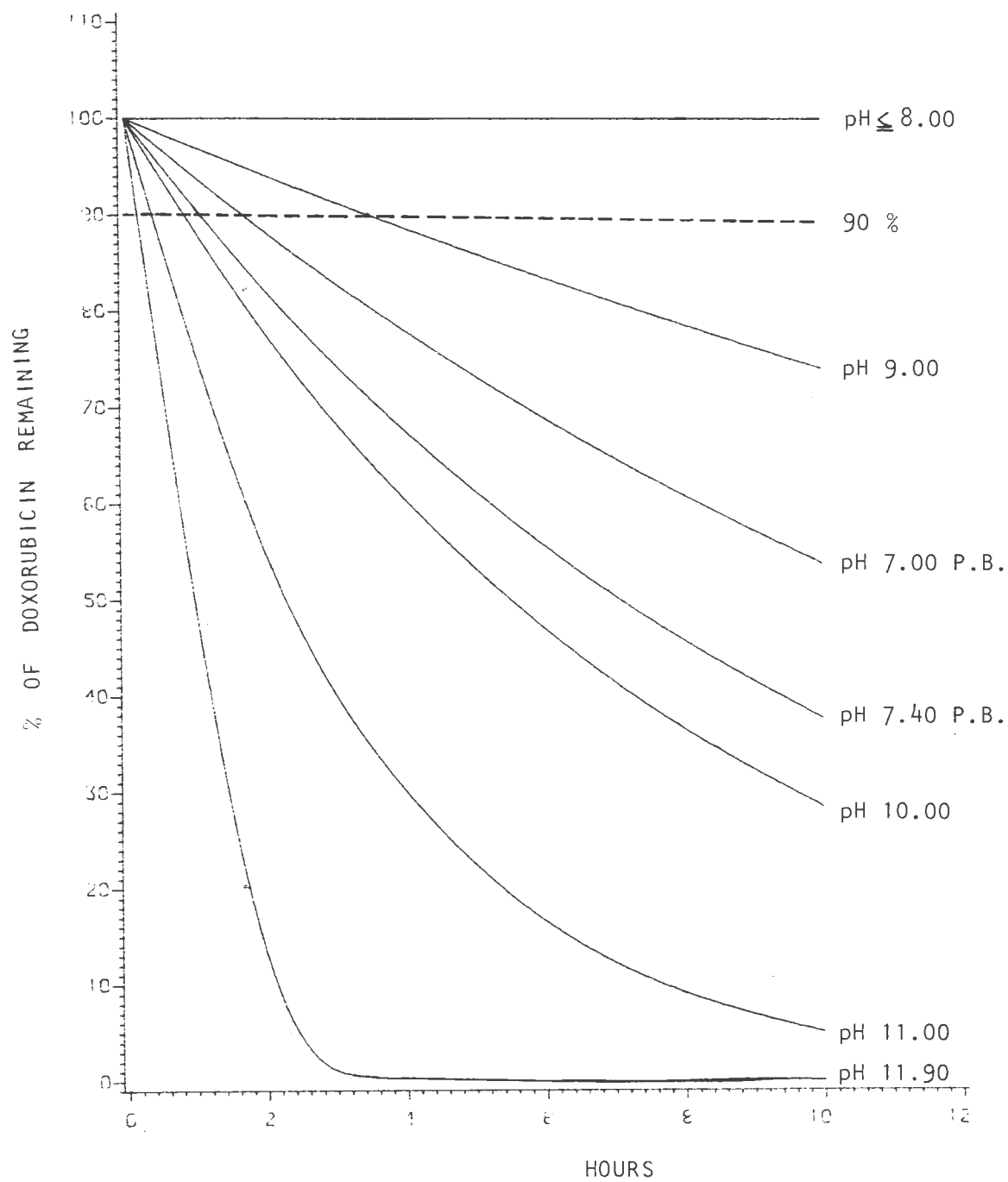


Figure 25. The effects of pH and buffering agents on doxorubicin stability. P.B. stands for phosphate buffer and the unspecified buffers were of the citrate-phosphate-borate buffer system.

sample group	thawing/freezing cycles	doxorubicin concentration (ng/ml)	% of DCX remaining
interval=1 day	1	753.2±30.1	100.4
	2	722.1±40.6	96.3
	3	685.0±34.2	91.3
	4	650.4±29.5	86.7
	5	619.3±37.1	82.6
	6	579.1±40.5	77.2
	7	542.8±21.4	72.4
	8	514.2±30.8	68.6
	9	486.5±29.2	64.9
	10	452.9±25.4	60.4
control group* (10)	0	721.5±40.1	96.1
interval=3 days	1	750.3±38.6	100.0
	2	713.3±32.5	95.1
	3	688.4±38.9	91.8
	4	650.6±28.6	86.7
	5	617.1±35.8	82.3
	6	578.6±42.1	77.1
	7	540.2±28.5	72.0
	8	511.6±35.1	68.2
	9	486.7±23.4	64.9
	10	451.8±29.6	60.2
control group* (30)	0	636.1±38.2	84.8
interval=5 days	1	747.6±27.1	99.7
	2	715.4±29.8	95.4
	3	680.2±41.1	90.7
	4	651.0±37.1	86.8
	5	622.5±40.4	83.0
	6	575.1±45.6	76.7
	7	537.7±32.2	71.7
	8	508.3±29.7	67.8
	9	489.1±35.4	65.2
	10	446.2±31.2	59.5
control group* (50)	0	531.2±27.4	70.8

* Control groups were the sample remained frozen for the days designated in the parentheses

Table 17. The concentration of doxorubicin in frozen plasma which experienced different freezing/thawing cycles

acceptable storage period, with no thawing permitted, was set to be 1 month according to these data from the control groups. Also observed from Table 17 was the more significant effect of freezing/thawing cycles than that of the time period between cycles. A graph of the freezing/thawing cycles versus doxorubicin concentration could further demonstrate this relationship (Fig. 26).

This discovery was very important in handling plasma samples and implied that a reasonable storage time of a month was tolerable as long as the repeated freezing/thawings were avoided. Eksborg et al. suggested that adsorption of doxorubicin onto the precipitates of these thawed samples might be the reason for degradation (52). Although precipitates did occur in thawed plasma, they could be easily disrupted and became homogenized by well stirring. The persistent precipitates ever existed was the plasma samples of more than three months old and had experienced at least 5 cycles of freezing/thawing. Therefore, reasonably fresh plasma (no more than 3 months old) was used in all spiked experiments.

Therefore, the most suitable storage conditions for plasma samples is to deep-freeze the samples until assay and repeated freezing/thawing should be avoided as much as possible. And it would be wise to perform the repeated

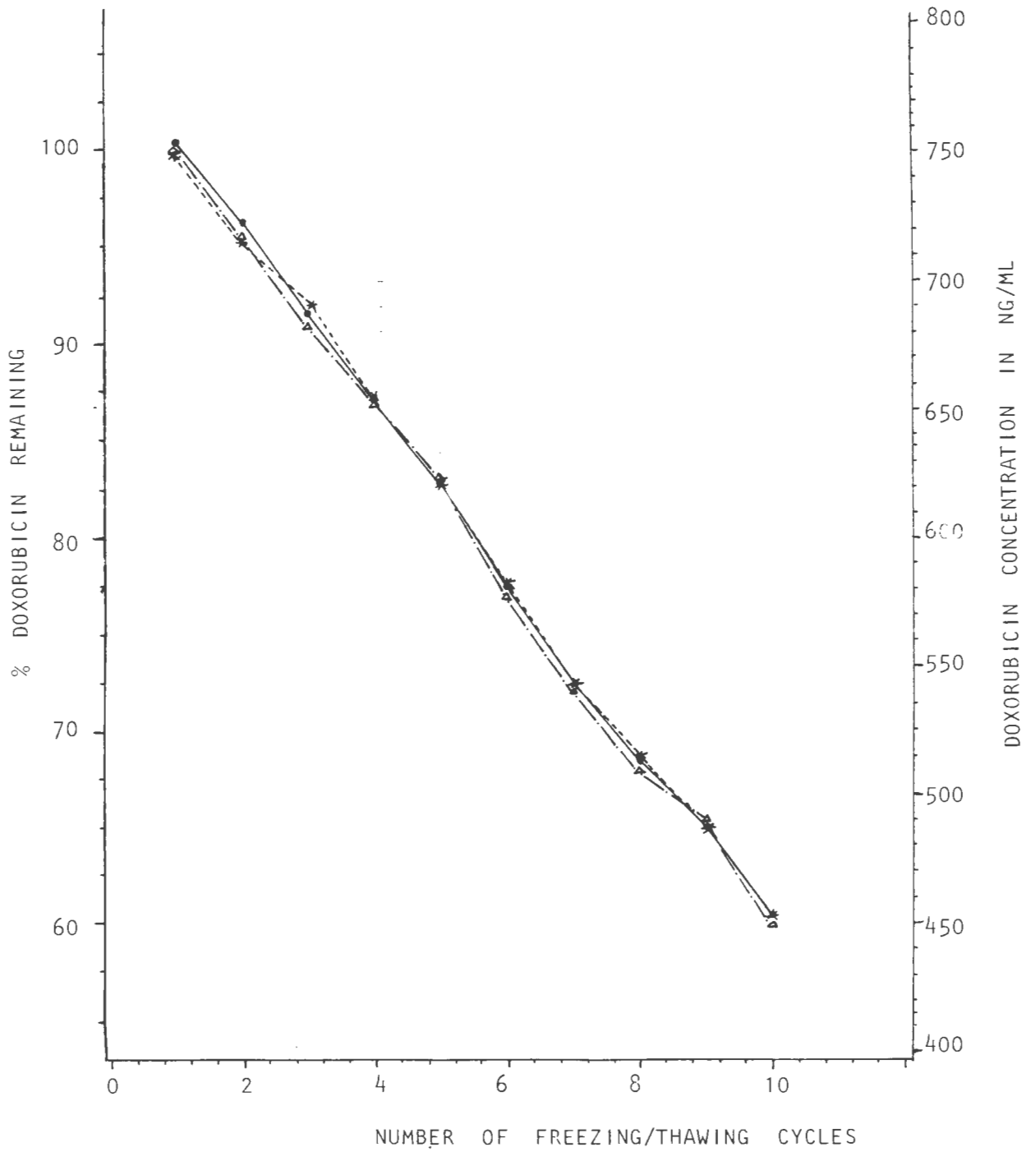


Figure 26. The effects of the number of freezing/thawing cycles on doxorubicin stability in frozen plasma. The time interval between freezing/thawing were 1(., —), 3(*, ---) and 5(Δ , -.-) days respectively.

assays within a week and confine the deep-freeze period to no more than 1 month.

D. Protein Binding

1. Direct Ultrafiltration Method

Direct ultrafiltration ('wash-out') method was first performed to determine the appropriate doxorubicin concentration in the reservoir cell that used in the continuous ultrafiltration. And 5 $\mu\text{g/ml}$ of doxorubicin turned out to be the minimal detectable concentration. Since only the unbound drug could pass the Diaflo membrane, the ultrafiltrate thus collected also provided a measurement of the fraction of doxorubicin bound to albumin.

In an ideal membrane, the Diaflo membrane should be non-retainable toward doxorubicin and concentration in the ultrafiltrate should be exactly the same as that in the filtration cell. But the highly stained Diaflo membrane indicated that there was some degree of membrane binding, and this binding could be quantified by the concentration difference between the ultrafiltrate and the filtration cell solution in the blank run. The concentration difference between ultrafiltrate and the filtration cell solution of a normal run (with 4 % albumin in the filtration cell), consequently represented not only the fraction bound to albumin but also the fraction bound to membrane.

consequently decreased the available surface area for doxorubicin binding (67). Thus, a competitive binding toward the Diaflo membrane was observed between doxorubicin and albumin. The membrane binding phenomenon usually could be lessened by increasing doxorubicin concentration in the filtration cell. Experiments were carried out with higher concentration (upto 20 $\mu\text{g/ml}$) but no significant improvement was detected. Therefore, no information regarding the fraction bound of doxorubicin was available from this direct ultrafiltration method. And this unusual result implied that doxorubicin may not be highly bound to albumin otherwise, the effect of membrane binding would have been insignificant.

2. The Continuous Ultrafiltration Method

Ultrafiltrates of a 'wash-in' (continuous) experiment were collected and assayed chromatographically. Small fractions of ultrafiltrate (0.5-1.0 ml) were collected for the first 15 ml and ultrafiltration was stopped after the collection of about 40 ml of ultrafiltrate. A blank run with no albumin in the filtration cell was performed to correct for membrane binding of doxorubicin.

For an ideal membrane, i.e., the dialyzable species penetrates unretarded through the membrane and does not

bind to the membrane (67), the following expression holds true in this case:

$$\ln \left(\frac{C_r}{C_r - C_f} \right) = \frac{V - V'}{V_0} \quad (2.1)$$

C_r : the concentration of drug in reservoir cell

C_f : the concentration of drug in ultrafiltrate

V : the cumulative volume of ultrafiltrate

V' : the apparent void volume of the system

V_0 : the average sample volume in the filtration cell during the run

This relationship shows that a plot of $\ln(C_r/(C_r-C_f))$ versus V should be a straight line with slope equal to $1/V_0$ and an intercept of $\ln(C_r/(C_r-C_f))$. Representative plots are shown in Fig 27. Although the above equation holds true for either a blank or normal run, the apparent void volume (V') and the sample volume of the filtration cell (V_0) could be better estimated from the blank run since less interference existed.

The sample volume of the filtration cell (V_0), calculated from the slope (slope= $1/V_0$) of the the blank run in this group, was 97.9 ml. This value was much higher

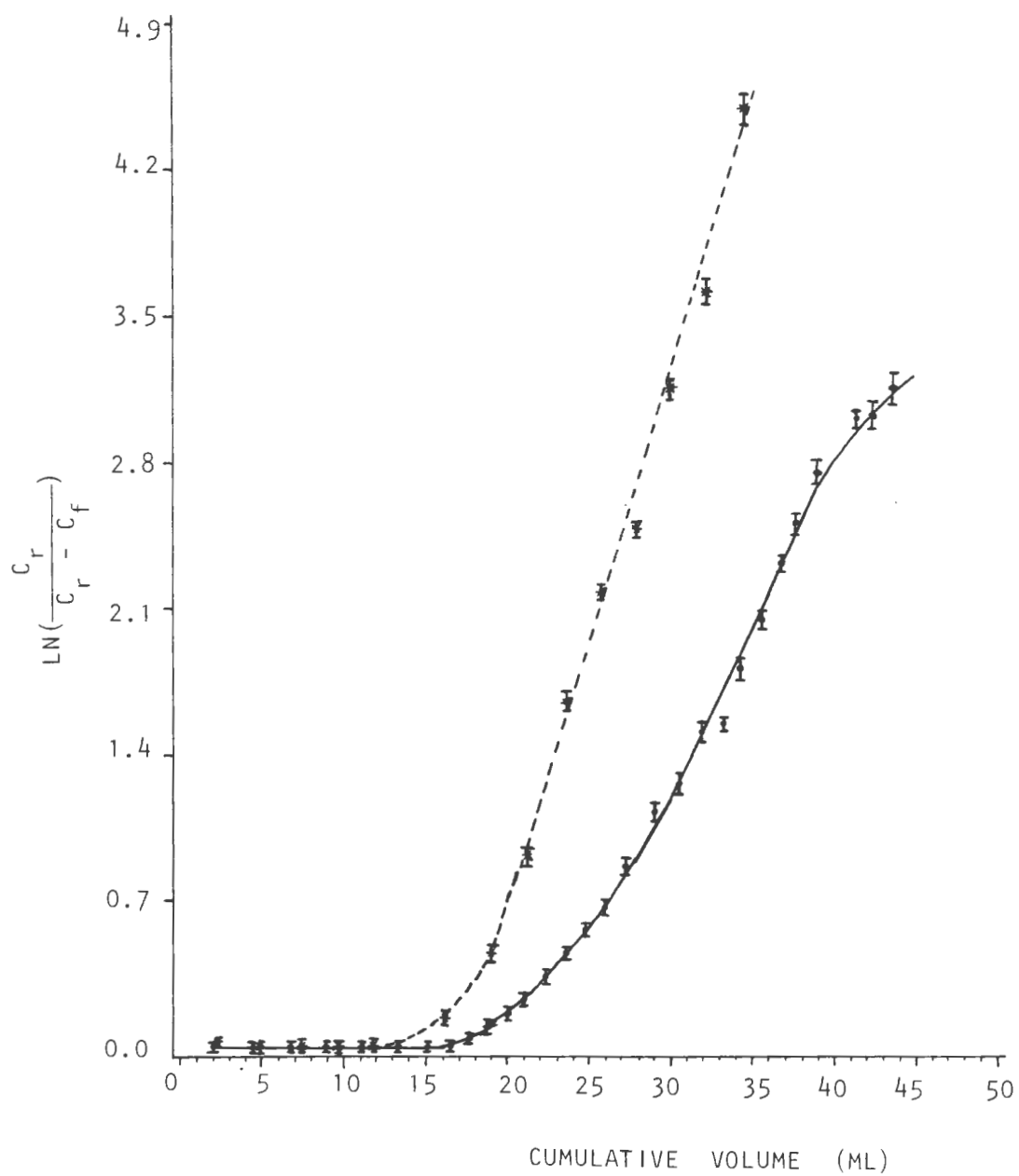


Figure 27. Ultrafiltration of doxorubicin ($20 \mu\text{g/ml}$) through the Diaflo PM-10 membrane at 10 p.s.i..
 Data were obtained with a blank run (albumin = 0) (*, ---) and with a normal run (albumin = 4%) (.,—).

than the observed value of 8 ml. Therefore, membrane binding was significant for doxorubicin. This membrane binding property could also be observed from the large apparent void volume ($V'=17.0$ ml) and the large area between sigmoid curves of the blank run and of the normal run. For doxorubicin, Diaflo membrane did not behave ideally and did retain doxorubicin to some degree, i.e., the reflection coefficient (σ) was not equal to zero. The reflection coefficient is defined as follows (67):

$$\sigma = 1 - (C'/C_f) \quad (2.2)$$

where c' is the free drug concentration in the filtration cell and C_f is the drug concentration in the ultrafiltrate. Accordingly, equation (2.1) should be modified as follows:

$$\ln \left(\frac{C_r}{C_r - C_f} \right) = \frac{V' - (1 - \sigma) V}{V_0} \quad (2.3)$$

The slope of the straight line from a blank run should then be $(1-\sigma)/V_0$. The reflection coefficient thus calculated, taking V_0 as the observed value of 8 ml, was 0.918. This value was much higher than those of Blatt et al., which ranging from 0.1-0.25 (67). Therefore, effort was made to decrease this membrane binding phenomenon.

Correction for membrane binding can be made, as suggested by Blatt et al., by either an increase in ratio of the sample volume (i.e., the volume of filtration cell) to membrane area or by an increase in the concentration of reservoir cell (67). Due to the fixed ratio of the sample cell and membrane area in the ultrafiltration unit, increment in doxorubicin concentration was made to decrease membrane binding. Higher concentrations of doxorubicin were prepared for the reservoir cell and the results showed in Fig. 28. From Fig. 28, the apparent void volume of the system were 12.20 ml, 21.08 ml, 22.91 ml respectively for reservoir concentration of 20, 10, 5 $\mu\text{g/ml}$. The reflection coefficient did decrease to 0.511 when reservoir concentration of doxorubicin was 20 $\mu\text{g/ml}$. This concentration of 20 $\mu\text{g/ml}$, however, was beyond the normal physiological range of doxorubicin (1 - 10 $\mu\text{g/ml}$) and it would be meaningless to perform such a study (15,44,45,79-81).

Three repeated runs of 10 $\mu\text{g/ml}$ were performed and the Scatchard plots of these data showed in Fig. 29. It was observed from these plots that there were more than one binding sites in albumin for doxorubicin. Therefore, the following equation would be adequate to express this type of binding (58,62):

$$r = \sum_{i=1}^n N_i \frac{K_i C_f}{1 + K_i C_f} \quad (2.4)$$

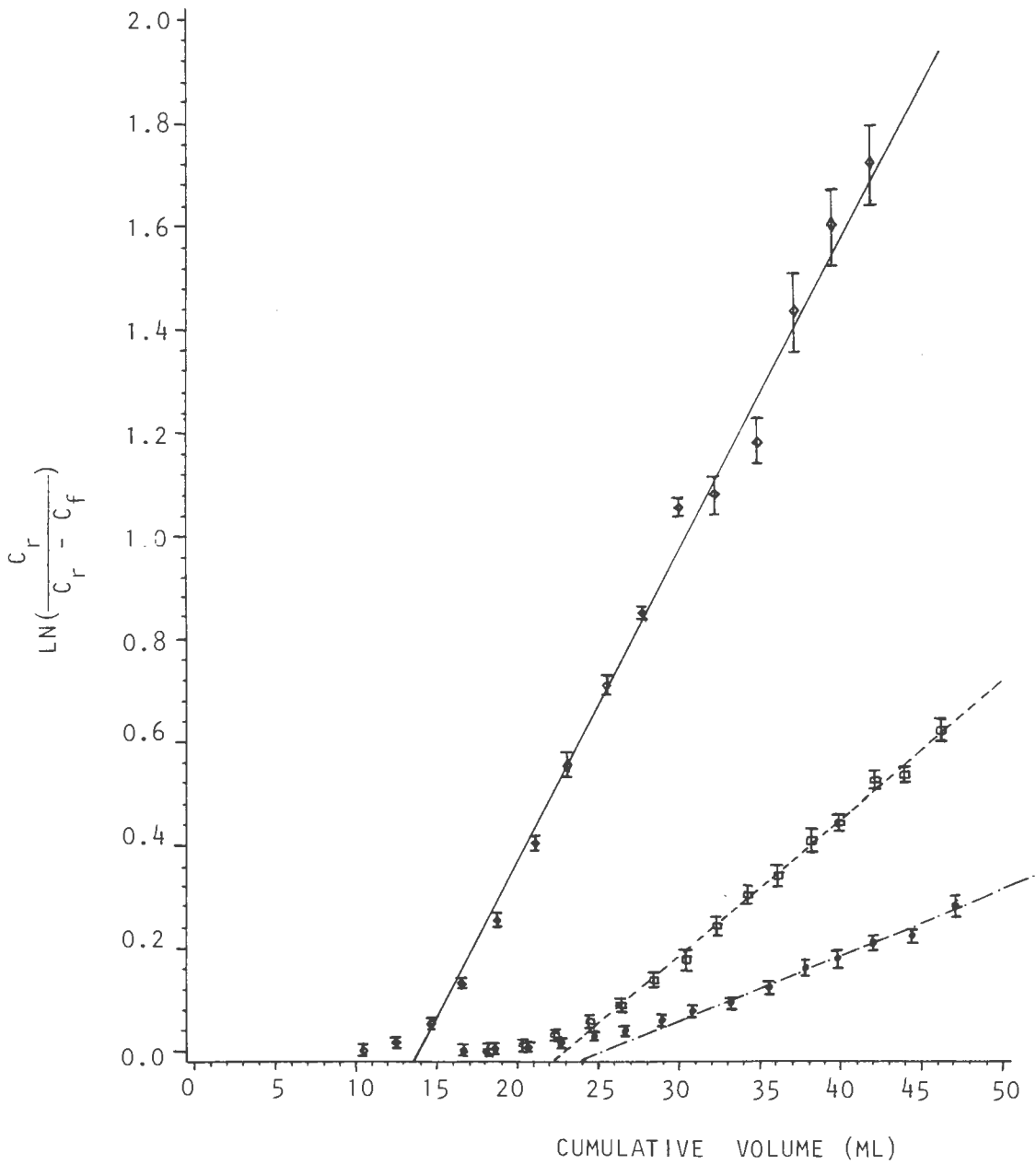


Figure 28. Ultrafiltration of doxorubicin through the Diaflo PM-10 membrane for various concentrations at 10 p.s.i.. Data were obtained from blank runs (albumin = 0) of 5, 10 and 20 $\mu\text{g}/\text{ml}$ of doxorubicin solutions.

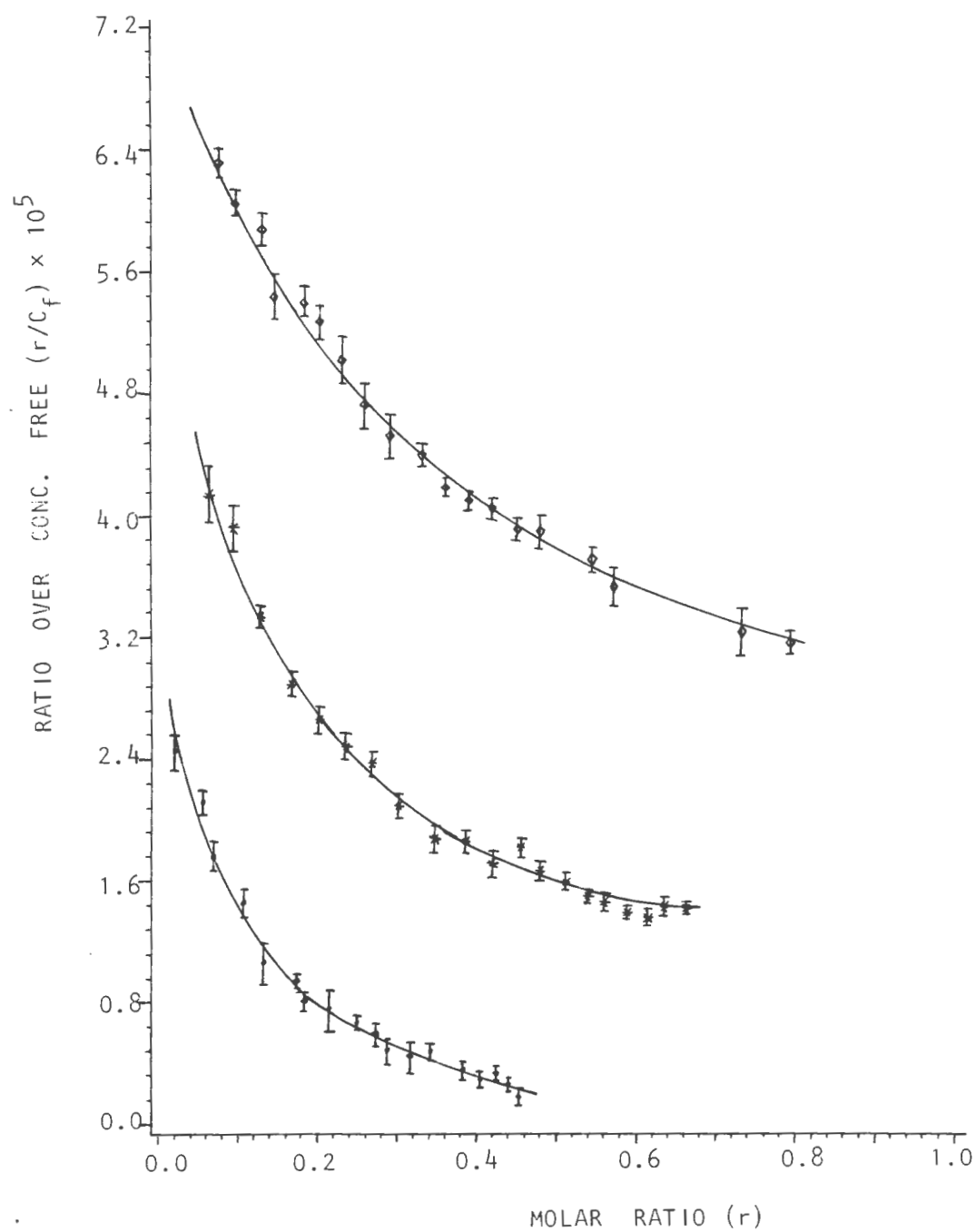


Figure 29. Scatchard plots of the albumin binding of doxorubicin (10 $\mu\text{g}/\text{ml}$) using the ultrafiltration method with the albumin concentration being 4 %

For doxorubicin, N_i , K_i should be the same for a fixed concentration of albumin. This was not the case in the curvilinear plots shown in Fig. 29.

The variation may result from the binding variation among the Diaflo membranes used. A protein binding study of methyl orange was performed under the same conditions. The Scatchard plots of these data showed in Fig. 30. The binding patterns changed, i.e., with different N_i and K_i , only when the concentration of albumin differed. Data of the same albumin concentration superimposed with each other regardless of the concentration of methyl orange. The reflection coefficient and void volume were 0.022 and 1.2 ml for curve A (0.1 % albumin solution) and 0.521 and 1.4 ml for curve B (0.2 % albumin solution). And it was confirmed that the much larger void volume of the doxorubicin data was resulted from the delayed elution of doxorubicin caused by membrane binding. And the higher values in the reflection coefficient in doxorubicin binding study was also attributed to membrane binding.

In summary, continuous ultrafiltration proved to be an inadequate method for studying the protein binding of doxorubicin. The Diaflo membrane showed such a large degree of binding toward doxorubicin that caused an inconsistency among data. And the higher doxorubicin

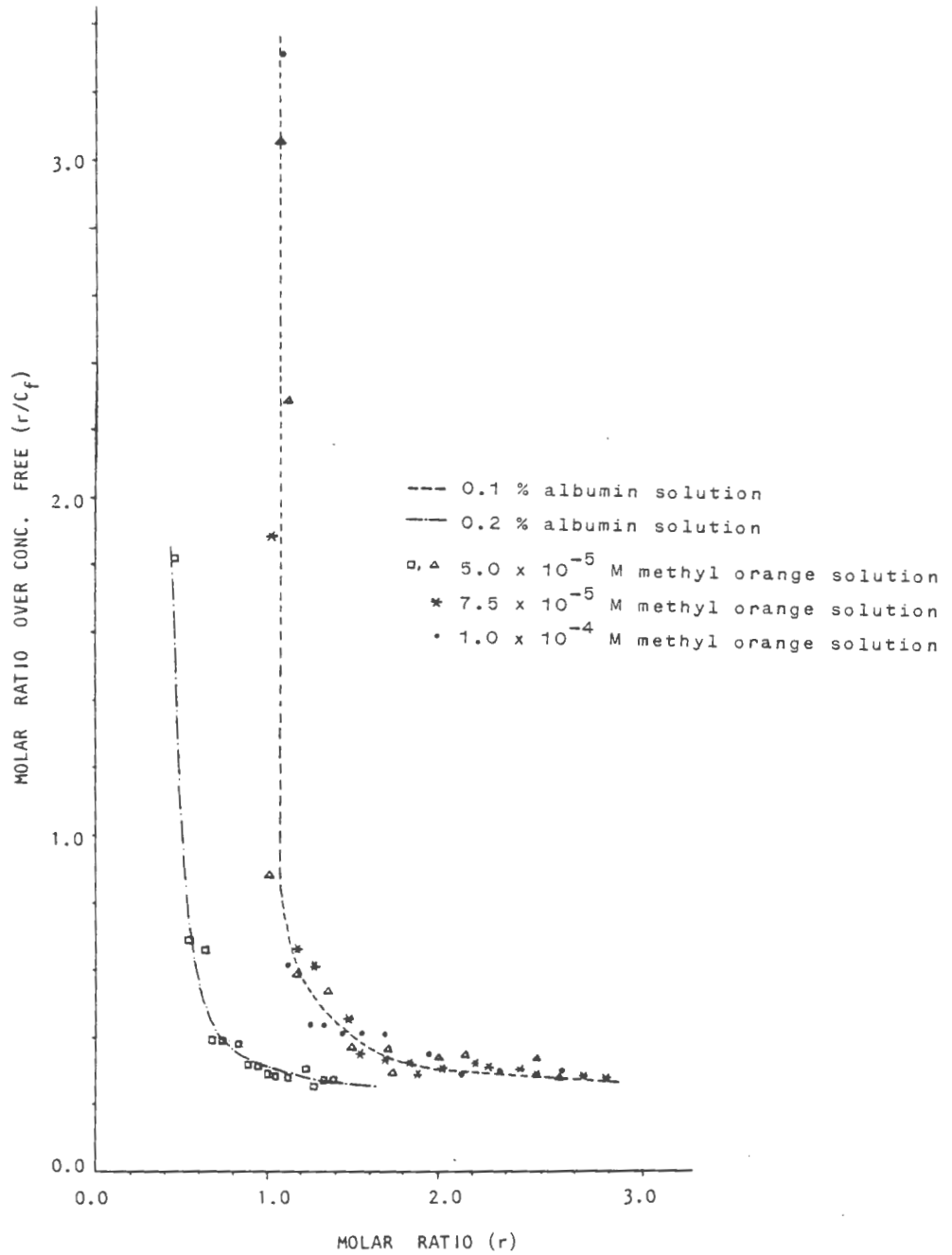


Figure 30. Scatchard plots of the albumin binding of methyl orange using the ultrafiltration method.

concentration that would have eliminated such phenomenon (20 $\mu\text{g/ml}$) was way beyond the range of doxorubicin levels in plasma samples (15,44,45,79-81).

The major obstacle of studying doxorubicin protein binding, therefore, was the binding to Diaflo membrane in the ultrafiltration method. A similar observation was made by Harris and Gross when a dialysis method was attempted to interpret doxorubicin protein binding (63). The curvilinear behavior on the Scatchard plot was difficult to interpret due to the binding of doxorubicin to cellulose dialysis bag. However, an ultracentrifugation method of 18 hours was performed and an extent of 50 % doxorubicin bound to human plasma was determined. Chan et al. re-analyzed the original Scatchard plot data of Harris and Gross and determined that the fraction bound in the therapeutic plasma concentration range was 0.9 rather than the original reported 0.5 (64). They did not offer specific reasons for such a modification and the resultant data of 0.9 fraction bound was, therefore, not convincing. And ultracentrifugation seemed to be the last resort for the determination of the fraction bound of doxorubicin to albumin solution.

3. The Ultracentrifugation Method

The results from the ultracentrifugation method showed that 70.5 ± 2.4 % of doxorubicin was bound to 4 % albumin. This value was obtained after the correction for non-specific adsorption of 3.6 % was made.

Although this result was obtained by covering only one concentration of doxorubicin solution it provided an initial estimation of the protein binding of doxorubicin. Thus, doxorubicin was not highly bound to albumin. And plasma levels or even tissue levels would not likely be influenced by changes of protein binding. Therefore, the possible role of plasma doxorubicin serving as a depot was excluded from this observation.

IV. CONCLUSIONS

1. The Sep-pak method was coupled with the reversed phase liquid chromatography and thus provided a sensitive, efficient reproducible and simple assay method for doxorubicin and its metabolites.
2. The Sep-pak method recovered 107.3 % of the spiked amount from plasma and 97.8 % from tissue samples.
3. Plasma samples required no preliminary treatment while tissue samples needed to be homogenized and freed from binding of doxorubicin to nuclear components by silver nitrate.
4. The Sep-pak method was superior to the conventional extraction method in efficiency (107.3 % vs. 86.1 %), processing time (1 minute vs. 30 minutes per sample), and ease of operation (one single procedure vs. three extractions).
5. The HPLC system consisted of a chromatographic column, a solvent delivery system, a fluorescence detector, a SC

102 active filter, an automatic sample processor and a data acquisition system. And various modes of HPLC, different in the column and mobile phase used, were under investigation. The HPLC system that employed a C-18 column and a mobile phase of 0.01 M H_3PO_4 in 40 % acetonitrile solution was good for stability studies of doxorubicin. The retention times of doxorubicin and daunorubicin were 3.93 and 5.34 minutes respectively, which provided a good resolution between these two compounds and saved the time and solvents of analysis. The HPLC system that used a C-18 column and a mobile phase of 0.01 M H_3PO_4 in 35 % acetonitrile solution was good for protein binding studies. This system provided a good resolution between interferences and doxorubicin and a short retention time (5.70 minutes for doxorubicin) to be economic of time and solvent of analysis. The HPLC system that used a C-18 column and a mobile phase of a mixture of 0.01 M $NH_4H_2PO_4$ solution and methanol (40:60) was used for the analysis of doxorubicin and its metabolites in biological samples. It provided a good resolution among doxorubicin and its metabolites, however, 68.02 % of sensitivity was simultaneously lost. A normal phase HPLC system was studied and offered no advantages over the systems mentioned above.

6. Pharmacokinetics of doxorubicin and its metabolites, using the developed assay method, were studied in human and rat plasma, and rat kidneys. The concentrations of doxorubicin have been demonstrated to be in good agreement with those of the literature and have a small variation in repeated assays. Pharmacokinetic parameters, such as initial concentration, volume of distribution, half-lives and microconstants (K_{12} , K_{21} and K_{el}), were obtained by AUTOAN and they showed good agreement with each other, particular in human plasma samples. The half-lives of α and β phases correlated well with literature data. However, a comparison of the pharmacokinetic parameters between the young and old rats failed to demonstrate significant effects of age on doxorubicin pharmacokinetics. These studies did demonstrate the successful application of coupling of the Sep-pak method and the reversed phase HPLC system.
7. Doxorubicin was more stable in acidic media. In a buffer system of citrate-phosphate-borate buffer, its stability dropped drastically around pH 11. The stability of doxorubicin was also influenced by the buffering agents used and the difference in rate constants may be 20-fold in different buffers of the same pH.

8. The recommended storage conditions for doxorubicin in plasma was to deep-freeze the samples until analysis and the repeated thawing/freezing should be avoided as much as possible. Plasma samples remained stable (i.e., degraded no more than 15 % of original doxorubicin) within one month and the length of the time intervals between thawings had no significant effects on doxorubicin degradation.

9. Ultrafiltration was found to be unsuitable for the protein binding study of doxorubicin. The high degree of membrane binding hindered the observation of fraction bound by the direct method and caused inconsistent data in the continuous method. But the ultracentrifugation method revealed that 0.7 fraction of doxorubicin was bound to plasma solution (4 % albumin in pH 7.4 phosphate buffer).

Future Studies

Several possibilities for future investigation have resulted from this work:

1. The stability of doxorubicin in tissue samples should be investigated. The suitable storage conditions for

tissue samples could be established only after the maximal storage period and the effects of thawing/freezing cycles on degradation have been determined.

2. The fraction bound of doxorubicin to albumin should be determined by the ultracentrifugation method with more than one concentrations in the physiological range. These experiments will buffer the possible variation in the fraction bound caused by difference in doxorubicin concentrations and, therefore, provides a more accurate data. Besides, it would be advantageous to obtain a series data of molar ratios at different free concentrations of doxorubicin and thus the more important binding parameters, such as the number of independent binding sites, the number of doxorubicin molecules binds to each site and the association constants, could then be determined.

3. A complete pharmacokinetic profile of doxorubicin in rats should be established by assaying the levels of doxorubicin and its metabolites in plasma, liver, kidney, spleen, lung and heart. After a detailed understanding of the kinetics of distribution and elimination of doxorubicin, a suitable pharmacokinetic model could then be established. By this

pharmacokinetic model, monitors of cardiac toxicities could be possibly established and suitable dose and schedule of administration can be determined to reach the predicted drug concentrations in plasma and tissues on an individual basis.

4. Larger sample size ($n > 3$) of rats of young, medium and old age should be included in the study of the effects of age on doxorubicin pharmacokinetics. Since there is an increasing proportion of the aged in total patient population, this pharmacokinetic study in geriatrics may provide better means to determine the dose and schedule of administration of doxorubicin.

APPENDIX

sample* (DOX/DAU) (ng/ml)	injection volume (μ l)	peak height ratio	coefficient of variation
20/100	200	0.1353 \pm 0.0059	4.34
40/100	200	0.2458 \pm 0.0262	10.66
60/100	200	0.5010 \pm 0.0085	1.69
100/100	200	0.6341 \pm 0.1000	15.77
150/100	200	0.8950 \pm 0.0167	1.86
200/400	100	1.2927 \pm 0.0722	0.94
300/400	100	0.1803 \pm 0.0113	0.61
400/400	100	1.9282 \pm 0.5233	2.71
500/400	50	3.0074 \pm 0.3020	10.04
600/400	50	3.2724 \pm 0.1593	4.87
800/400	50	4.3371 \pm 0.1067	2.46
1000/400	50	5.0563 \pm 0.1565	3.09
1200/400	25	7.0102 \pm 0.1808	2.58
1600/400	25	8.8926 \pm 0.1562	1.76
2000/400	25	9.7910 \pm 0.3743	3.86

* The samples are expressed as the concentration of doxorubicin (DOX) and daunorubicin (DAU) in ng/ml

Table 1. The peak height ratios of the standard curve of spiked plasma samples by organic extraction method. The HPLC system consisted of a C-18 column, a mobile phase of 0.01 M H_3PO_4 in 32 % acetonitrile and a flow rate of 1.0 ml/min

sample* (DOX-DAU) (ng/ml)	injection volume (μ l)	peak height ratio	coefficient of variation
10-200	200	0.1617 \pm 0.0132	8.20
30-200	200	0.4613 \pm 0.0438	9.50
50-200	200	0.7497 \pm 0.0380	5.07
75-200	100	1.1253 \pm 0.0195	1.74
100-200	100	1.5525 \pm 0.0252	1.63
200-200	50	2.6621 \pm 0.0679	2.55
300-200	50	4.7691 \pm 0.2572	5.39
400-800	40	6.4345 \pm 0.2454	3.81
500-800	25	7.1413 \pm 0.0794	1.11
600-800	25	8.8011 \pm 0.1483	1.68
700-800	25	9.5400 \pm 0.0400	0.42
800-800	10	11.1401 \pm 0.1547	1.39
900-800	10	12.0020 \pm 0.4290	3.57
1000-800	10	13.6960 \pm 0.7998	5.84

* The samples are expressed as the concentration of doxorubicin (DOX) and daunorubicin (DAU) in ng/ml

Table 2. The peak height ratios of the standard curve of spiked plasma samples by Sep-pak method. The HPLC system consisted of a C-18 column, a mobile phase of 0.01 M H_3PO_4 in 32 % acetonitrile and a flow rate of 1.0 ml/min.

sample* (DOX-DAU) (ng/sample)	injection volume (μ l)	peak height ratio	coefficient of variation
50-800	100	0.2308 \pm 0.0057	2.46
100-800	100	0.4446 \pm 0.0205	4.62
200-800	100	0.7579 \pm 0.0172	2.26
400-800	75	1.3191 \pm 0.0612	4.64
600-800	75	1.1729 \pm 0.0408	2.36
800-800	50	2.3547 \pm 0.7794	7.62
1000-800	50	2.5961 \pm 0.0998	3.84
1200-800	40	3.3504 \pm 0.1051	3.14
1400-800	30	4.7392 \pm 0.1543	3.26
1600-800	30	5.1763 \pm 0.2663	5.14
1800-800	25	5.9337 \pm 0.3815	6.43
2000-800	25	7.4022 \pm 0.6456	8.72

* The samples are expressed as the concentration of doxorubicin (DOX) and daunorubicin (DAU) in ng/sample. Since the final sample volume was approximate 1 ml, this expression was similar to that used for the the plasma samples.

Table 3. The peak height ratios of the standard curve of spiked liver samples by Sep-pak method. The HPLC system consisted of a C-18 column, a mobile phase of 0.01 M $\text{NH}_4\text{H}_2\text{PO}_4$ solution and methanol (40:60) and a flow rate of 1.0 ml/min.

sample* (DOX-DAU) (ng/sample)	injection volume (μ l)	peak height ratio	coefficient of variation
30-1000	120	0.0627 \pm 0.0060	9.54
100-1000	100	0.1666 \pm 0.0106	6.39
200-1000	100	0.4308 \pm 0.0128	2.96
400-1000	100	0.7896 \pm 0.0169	2.14
800-1000	50	1.2831 \pm 0.0159	1.24
1200-1000	30	1.8715 \pm 0.0376	2.01
1600-1000	20	2.4533 \pm 0.1242	5.06
2000-1000	20	3.1644 \pm 0.0518	1.64
2400-1000	14	3.7609 \pm 0.0912	2.43
2800-1000	14	4.8768 \pm 0.2053	4.21

* The samples are expressed as the concentration of doxorubicin (DOX) and daunorubicin (DAU) in ng/sample.

Table 4. The peak height ratios of the standard curve of spiked kidney samples by Sep-pak method. The HPLC system consisted of a C-18 column, a mobile phase of 0.01 M $\text{NH}_4\text{H}_2\text{PO}_4$ solution and methanol (40:60) and a flow rate of 1.0 ml/min.

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