Stability of anthracycline cytotoxic agents in solution and infusion fluids.

by

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Submitted for the degree of Master of Philosophy

The University of Aston in Birmingham

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Information on the stability of doxorubicin and daunorubicin is available in the literature but data are limited and contradictory. In addition few data have been published for epirubicin. The aim of this study was to more clearly define the stability of these drugs in order to clarify the literature data. Reversed-phase, stability-indicating HPLC assays were developed and used to investigate the effect of pH on the rate and mechanism of degradation by construction of a pH profile for each drug. Degradation kinetics were studied in fluorescent light and long term stability investigated in infusion fluids. At concentrations greater than 500µg/ml no special precautions were necessary to protect freshly prepared solutions of these drugs from light. Below 100µg/ml photolysis was rapid but could be prevented by storage in amber glass or by covering the containers with aluminium foil. The rate of degradation was strongly influenced by pH and was catalysed in the presence of buffers. In buffers ($pH < 4$) epirubicin was more stable than doxorubicin or daunorubicin but in buffers ($pH \geq 4$), dextrose 5%, pH 4.36, and sodium chloride 0.9%, pH 5.2, this stability difference was not apparent. Maximum stability was observed between pH 4.0 and 5.0. In buffers ($pH \geq 7$) and sodium chloride 0.9%, pH 6.47, daunorubicin was more stable than doxorubicin or epirubicin. At concentrations less than 100µg/ml drug loss due to adsorption onto glass was appreciable. In PVC minibags, in addition to adsorption, a slow migration into the plastic matrix appeared to occur which was dependent on the properties of the drug, the pH of the vehicle, the surface area of PVC and the storage temperature. At concentrations greater than 500µg/ml, where adsorption (as a proportion of the total amount) was negligible doxorubicin, daunorubicin and epirubicin were stable in glass and plastic for at least two weeks and six weeks respectively.

Keywords:

doxorubicin
daunorubicin
epirubicin
stability
degradation

Acknowledgements.

I should like especially to thank Dr's W J Irwin and D K Scott for their invaluable guidance and supervision and for their constant encouragement throughout the experimental work and during the preparation of this thesis.

I also wish to thank the West Midlands Regional Health Authority for their sponsorship, without which this work would not have been possible, and especially Dr T Bradley for his encouragement and advice.

My thanks to my husband, Mike, for his patience and support over the past three years and for the many hours spent in the preparation of the figures in this thesis.

Finally, I would like to extend my thanks to all the pharmacy staff at the General and Queen Elizabeth Hospitals for the interest they have shown in my research, and especially to Mr S Potter for his support during the last twelve months and Mr A Auld for his help with the word processor.

Contents. PAGE

List of Tables.

List of figures. PAGE

Figure

Chapter One

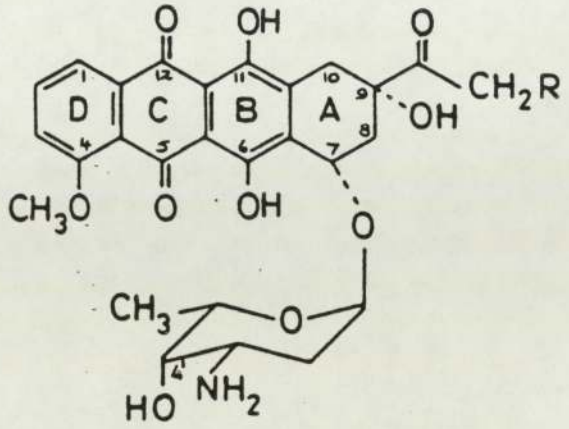
Introduction

1.1. Structure.

The anthracycline cytotoxic agents which include doxorubicin, daunorubicin and epirubicin consist of an amino sugar moiety linked through a glycosidic bond to the C7' of a tetracyclic aglycone. The amino sugar is an important structural requirement for bioactivity and modifies the solubility properties of the water-insoluble aglycone portion of the molecule [1].

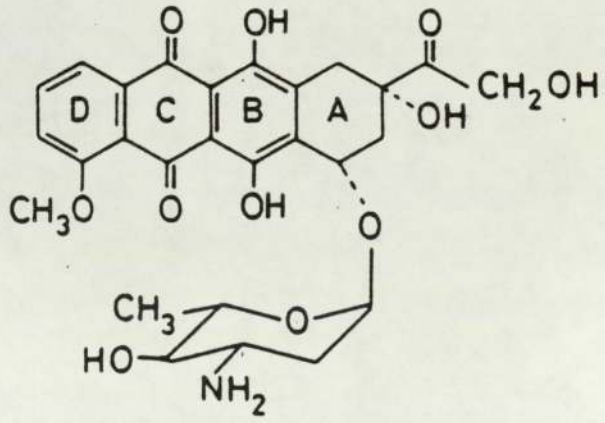
Doxorubicin differs from daunorubicin by a single hydroxyl on C14 [2] (Figure 1.1a). Despite this minor variation in chemical structure there is a marked difference in their antitumour activity [3]. Epirubicin is a semi-synthetic analogue of doxorubicin in which the stereochemistry of the hydroxyl group at C4' has been inverted to give an equatorial, rather than an axial, orientation (Figure 1.1b) [3,4,5]. The two are otherwise identical. The formulations of the anthracyclines which were used in the experimental work are shown in Table 1.1. Lactose is included in the formulation of doxorubicin and epirubicin and mannitol is included in the formulation of daunorubicin. These agents are present as excipients.

Doxorubicin has recently been re-formulated as a rapid dissolution preparation. In addition to the constituents shown in Table 1.1 each vial now contains methyl-hydroxybenzoate 0.02% [6]. The inclusion of methyl-hydroxybenzoate, in this sub-preservative concentration, prevents gel formation which is a particular problem after reconstitution with sodium chloride [7].



(1) doxorubicin R = OH
(2) daunorubicin R = H

Figure 1.1.a. Structure of doxorubicin and daunorubicin.



(3)

Figure 1.1.b. Structure of epirubicin.

DRUG	PRESENTATION	METHOD OF RECONSTITUTION
Adriamycin (doxorubicin)	Sterile, pyrogen free, orange-red, freeze-dried, powder, Vials containing 10mg or 50mg of doxorubicin hydrochloride with lactose.	Add 5ml or 25ml of Water For Injections to produce a solution of 2mg/ml. (pH approx. 6.0)
Pharmorubicin (epirubicin)	Sterile, pyrogen free, orange-red, freeze-dried powder, Vials containing 10mg or 50mg of epirubicin hydrochloride with lactose.	Add 5ml or 25ml of Water For Injections to produce a solution of 2mg/ml. (pH approx. 6.0)
Cerubicin (daunorubicin)	Sterile, pyrogen free, orange-red, lyophilized powder, Vials containing 20mg of daunorubicin hydrochloride with mannitol.	Add 4ml of Water For Injections to produce a solution of 4mg/ml. (pH approx. 6.0)

Table 1.1. Formulations of the anthracyclines used in the experimental work.

1.2. History.

1.2.i. Discovery and isolation of Daunorubicin.

Daunorubicin was discovered independently in 1963 by two groups working at Farmitalia and Rhone-Poulenc [8]. Initially, daunorubicin was isolated from cultural broths of Streptomyces peucetius [8,9,10].

Fermentation titres quoted in the patent literature ranged between 60 to 70mg per litre. By development of improved strains, extraction methods and purification procedures the yield was increased, and is now probably in excess of 500 to 1000mg per litre, although current titres in production-scale fermentations are not released by Rhone-Poulenc or Farmitalia [8].

Daunorubicin is primarily present as higher glycosides, such as the baumycins, in the fermentation broth [11]. A hydrolysis step was included in the extraction process to convert these glycosides to daunorubicin [8]. Finally, lyophilization of the resultant aqueous solution yielded daunorubicin hydrochloride of approximately 95% purity [12].

1.2.ii.Development and isolation of Doxorubicin.

While antimitotic studies were being conducted with daunorubicin, microbiologists attempted to modify the genetic code of Streptomyces peucetius in order to alter its metabolic pathways and obtain new compounds [13]. Analysis

of metabolic products of a strain named Streptomyces peucetius var. caesius revealed the presence of 14-hydroxydaunorubicin (doxorubicin) which was extracted from a cultural broth in a similar way to daunorubicin [12].

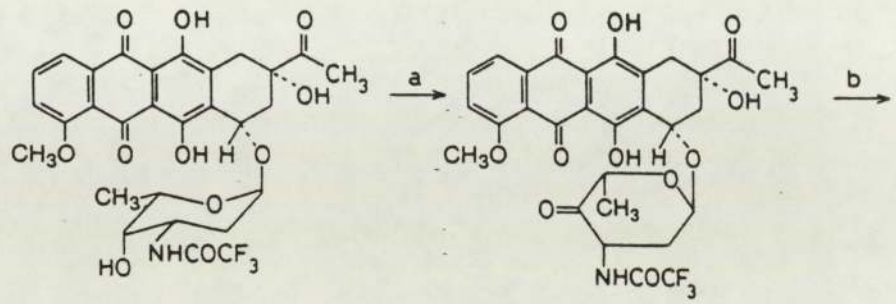
1.2.iii. Total synthesis.

The low yield obtained in the extraction of doxorubicin and daunorubicin from cultural broths [14] aroused interest in the total synthesis of the anthracyclines which could provide an alternative source of this drug and generate new antitumour agents. Two different methods of daunomycinone synthesis have been published [15,16]. L-Daunosamine has been synthesized stereospecifically from L-rhamnose [17] and D-mannose [18]. The formation of glycosidic bonds between daunomycinone and daunosamine and the chemical conversion of daunorubicin to doxorubicin has provided total synthesis of these compounds in a formal sense [19]. Due to the complexity of the synthetic methods, at present, daunorubicin is still prepared from cultures of Streptomyces coeruleorubidus. After purification by ion-exchange chromatography and extraction with methylene chloride, crude daunorubicin is dried and recrystallized [20]. Doxorubicin is prepared by fermentation, separation and purification of daunorubicin. Conversion to doxorubicin is achieved through 14-halogenation, hydrolysis and hydroxylation of the 14-carbon atom of daunorubicin [21].

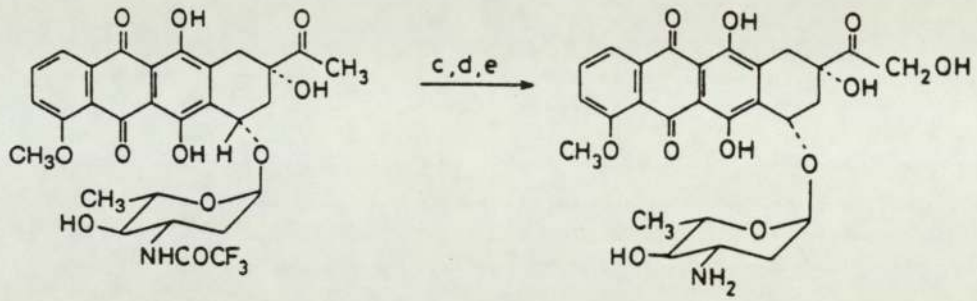
1.2.iv. Development of Epirubicin.

The structure-activity relationships of the anthracyclines have been extensively studied in order to identify the structural requirements for antitumour efficacy and to eliminate the features responsible for toxic effects [22]. Analogues of doxorubicin modified in the daunosamine moiety were of particular interest as tissue distribution, cellular uptake and intracellular distribution of the anthracyclines depends on the structure and stereochemistry of the carbohydrate side-chain [3].

Epirubicin is an analogue of doxorubicin in which the stereochemistry at the hydroxyl group bearing C4' has been inverted to give an equatorial orientation [3,4,5]. The synthesis of epirubicin, which is shown in Figure 1.2, involves an oxidation reaction followed by a regio- and stereo-selective reduction. The starting material, N-trifluoroacetyldaunorubicin (4), obtained by N-trifluoroacetylation of daunorubicin (2), is oxidized with dimethylsulphoxide, DMSO, at -70° C, in the presence of trifluoroacetic anhydride to yield the ketone derivative (5). 4'-Epi-N-trifluoroacetyldaunorubicin (6) is then produced by treatment with sodium borohydride at -30° C. Epirubicin (3) is obtained by following the method used for chemical transformation of the side chain of daunorubicin to that of doxorubicin [23,24].



(4) (5)



(6) (3)

Figure 1.2 . Synthesis of epirubicin (from reference [1]).

Reagents and conditions:

a) $(CH_3)_2SO$, $(CF_3CO)_2O$, Et_3N, CH_2Cl_2, $-70^{\circ}C$;
b) $NaBH_4$, CH_3OH, $-30^{\circ}C$;
c) 0.1M NaOH, $0^{\circ}C$;
d) Br_2, CH_3OH-dioxane, HBr;
e) HCOONa.

1.3. Use, clinical application and administration.

Doxorubicin has been used successfully to produce regression in a wide range of neoplastic diseases including; acute leukaemia, lymphomas, soft tissue and osteogenic sarcomas, paediatric malignancies and adult solid tumours; in particular, breast and lung cancers [2,14,25,26]. Doxorubicin is used as a single chemotherapeutic agent and in combination with other cytotoxics in numerous treatment schedules [27].

Dosage is usually calculated on the basis of body surface area. When doxorubicin is used as a single agent a dose of 60-75mg/m^2 is given every three weeks. In combination regimes, with other cytotoxics which possess overlapping toxicity, the dose is usually reduced to 30-40mg/m^2 every three weeks. In a current breast cancer trial three-weekly single-agent doxorubicin therapy (50mg/m^2) is being compared with either three-weekly epirubicin (75mg/m^2) or mitoxantrone (14mg/m^2). Three-weekly combination therapy of doxorubicin (40mg/m^2), cyclophosphamide (500mg/m^2) and methotrexate (40mg/m^2) is being compared with mitoxantrone (12mg/m^2), cyclophosphamide (500mg/m^2) and methotrexate (40mg/m^2) or with epirubicin (50mg/m^2), cyclophosphamide (500mg/m^2) and methotrexate (40mg/m^2). Alternatively, the three-weekly dose of doxorubicin may be divided over three successive days (20-25mg/m^2 on each day). Doxorubicin may also be administered on a weekly basis (20mg/m^2).

Doxorubicin is most frequently administered by the

intravenous route. The reconstituted solution is added, over two or three minutes, to the tubing of a free-running intravenous infusion of either dextrose 5%, sodium chloride 0.9% or dextrose 4% with sodium chloride 0.18%. This technique of administration minimises the risk of thrombosis or extravasation which can lead to severe cellulitis or vesication. Intravesical administration has been used for the treatment of transitional cell carcinoma, papillary bladder tumours and carcinoma-in-situ. This allows direct exposure of tumours to the drug and avoids systemic side effects [27].

Daunorubicin is indicated in the treatment of acute leukaemia [28-32]. In acute myeloblastic leukaemia (AML) it is used, either alone or in combination with other cytotoxics, at all stages of disease development. For AML the manufacturers recommend a dose of about 2mg/kg which is repeated at four to seven day intervals. Dosage adjustment is made according to response and haematological parameters. In combination regimes dosages should be suitably adjusted [33]. Currently, the ninth acute myeloblastic leukaemia (AML 9) trial is in progress. This trial aims to compare the treatment used in the previous trial (AML 8) (daunorubicin 50mg/m^2 on day 1, cytosine 100mg/m^2 and 6 thioguanine 100mg/m^2 both on days 1 to 5) with (daunorubicin 50mg/m^2 , on days 1,3 and 5, cytosine 100mg/m^2 and 6 thioguanine 100mg/m^2 on days 1 to 10). Due to availability of other treatments and particularly because of its high toxicity, daunorubicin is indicated chiefly for cases of AML that are

resistant to treatment with other drugs [33]. In acute lymphoblastic leukaemia (ALL), doses of 1mg/kg may be repeated, at one to four day intervals, according to tolerance and effect. In combination regimes the dosage is suitably reduced [33].

Epirubicin, as a single agent, has produced regression in a wide range of neoplastic diseases including breast, ovarian, gastric and colorectal carcinomas, lymphomas, leukaemias and multiple myeloma [4,34,35]. When epirubicin is used as a single agent a dose of 75-90mg/m^2 is given every three weeks. In combination regimes the dose is reduced to 50mg/m^2 every three weeks [36]. Epirubicin has also been used in combination chemotherapy. Tumour responses to epirubicin are currently being compared to those already obtained for doxorubicin [36].

Daunorubicin and epirubicin are only administered by the intravenous route. The manufacturer recommends addition of the reconstituted solutions into the tube of a freely running infusion of sodium chloride 0.9%, over three to five minutes, for the reasons outlined for doxorubicin [33,36].

1.4. Mechanism of action.

Deoxyribonucleic acid (DNA) has been considered to be the primary target for the cytotoxic action of the anthracyclines [37]. Doxorubicin does bind to double-stranded DNA by intercalation with reasonably high affinity [38,39]. After insertion into the double helix three types of interaction are responsible for stabilization of the

resultant complex. These involve electrostatic attraction between the protonated cationic group of the amino sugar residue and the ionized phosphate groups, hydrogen bonding and intercalation which is sustained by weak hydrophobic stabilization between the intercalating molecule and the adjacent base pairs [40]. The effect of intercalation is primarily blockage of DNA, RNA and protein synthesis but fragmentation of DNA and inhibition of DNA repair also occur [2,41].

A review of the literature reveals no obvious relationship between the inhibition of nucleic acid synthesis and cytotoxicity. Analogues have been synthesized which do not bind to DNA but which are cytotoxic to cells. N- trifluoroacetyl-doxorubicin-14-valerate is an active anthracycline with little or no ability to bind to DNA [37]. Tritton [37] presented evidence that the cell surface is the target for the action of doxorubicin. Doxorubicin was covalently bound to large insoluble polymeric beads by an N-alkyl carbamate linkage and unattached drug removed by washing. Murine L1210 cells (15μm diameter) were then exposed to either free drug, bound drug or unbound polymer. Since the support was larger than the cells (40 to 210μm diameter), the drug could not penetrate the cytoplasm or nucleus. Any cytotoxic effect under these conditions would be direct evidence that DNA intercalation or any other intracellular binding is not essential for expression of the pharmacological activity of doxorubicin.

Results showed that both free drug, as expected, and bound drug caused a decrease in survival of the cells. The

cell contents were analysed for drug and metabolites by high-performance liquid chromatography (HPLC) to ascertain if doxorubicin was released from the polymer. Although some drug was released on the first exposure of the complex to the cells on all following exposures the complex remained cytotoxic without release of doxorubicin. Since polymer alone was not cytotoxic it was concluded that doxorubicin must be the active component and the site of action was at the cell surface. The research of other authors has strengthened this membrane action hypothesis. Doxorubicin has been bound irreversibly to reactigel, dextran, sephadex and polyvinyl alcohol by a variety of coupling methods and still remained active, even though the immobilized drug was not transported into the cell [42].

1.5. Pharmacokinetics.

The pharmacokinetics of doxorubicin, daunorubicin and epirubicin after intravenous administration, can be described by a three-compartment open model and hence a triphasic disappearance curve [5,43-47] using the equation:

$$Cp(t) = Ae^{-\alpha t} + Be^{-\beta t} + Ce^{-\gamma t}$$

where Cp(t) is the concentration at time t

A,B and C represent the three compartments

α represents the distribution phase

β represents the metabolic phase

γ represents the elimination phase

A typical time course of plasma levels of doxorubicin after an intravenous bolus injection of 40mg/m^2 is shown in Figure 1.3 (below).

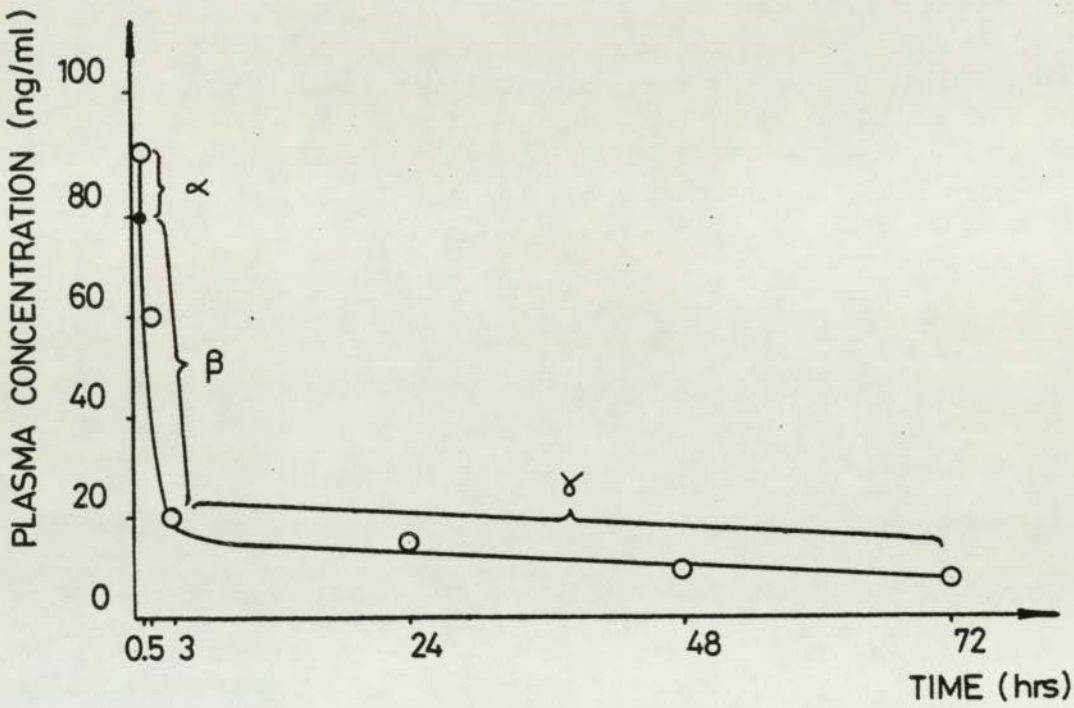


Figure 1.3. Plasma concentration time profile for doxorubicin (from Van Lancker et al [48]).

Some of the pharmacokinetic parameters of doxorubicin, daunorubicin and epirubicin are shown in Table 1.2. The initial phase ($t1/2\alpha$), which is much less than 30 minutes, is considered to represent tissue uptake and extensive protein binding, the second, ($t1/2\beta$), which is approximately 2.5 hours, hepatic metabolism and the slow final phase, ($t1/2\gamma$), which is approximately 70 hours, gradual release of the drugs from multiple binding sites [2]. The large volume of distribution (Vd_{SS}) indicates significant tissue distribution and explains the apparent discrepancy between the quite efficient plasma clearance (Clpl) and the slow elimination of these drugs [44].

PARAMETER	EPI	DOX	DAU
$t_{1/2}\alpha$ (mins)	3.1-4.8	4.4-4.6	3.4-5.4
$t_{1/2}\beta$ (hours)	1.3-2.6	1.6-2.5	3.5
$t_{1/2}\gamma$ (hours)	30-38	41-70	14.4-16.4
Vd_{SS} (l/m^2)	1430	960	1297
Cl_{pl} (l/min)	0.88-1.5	0.883	0.740

Table 1.2. Some pharmacokinetic parameters of epirubicin, doxorubicin and daunorubicin (from Cersosimo & Hong [5], Rahman et al [46], Weenen et al [47], Natale et al [49] and Oosterbaan et al [50].

1.5.i. Absorption and Distribution.

Daunorubicin, doxorubicin [14] and epirubicin are not absorbed orally due to hydrolysis in the gastrointestinal tract [51]. For this reason administration is by the intravenous route.

There are several potential mechanisms of drug transport into cells. Small polar molecules can diffuse through water-filled membrane pores whereas lipophillic drugs may diffuse through the lipids in the cell membrane. Alternatively, passage into the cell can occur via active or passive carrier-mediated transport systems [52]. Dano [53] and Skovsgaard [54] suggested the existence of carrier-

mediated facilitated transport systems for the anthracyclines. However, Dalmark [55] and Brown [14] contended that anthracycline influx occurred by simple diffusion of the electrochemically neutral molecule through the lipid domain in the cell membrane. Conversely, the efflux of anthracyclines is energy dependent and strongly influenced by the sodium ion gradient [22,56].

The rate of uptake into the cell is determined by the hydrophobicity, the pKa of the drug and the pH gradient across the membrane. An increase in the hydrophobicity of the drug leads to an increase in the rate of uptake. A decrease in the pKa of the drug has also been quoted to increase the rate of entry into cells. The lower the intracellular pH, with respect to the extracellular pH, the greater the rate of entry into the cell [22]. The partition coeffients and pKa values and some other properties of doxorubicin, daunorubicin and epirubicin are shown in Table 1.3.

The degree of passive diffusion into the cell is also determined by the membrane thickness, the total surface area of membrane exposed to the drug, the duration of drug cell contact and the membrane permeability [3]. In summary, passive diffusion occurs until the concentration of unionized drug on each side of the cell membrane is equal. The rate of accumulation is a balance between influx and efflux. The amount of accumulation depends upon removal of free drug, by binding to cellular macromolecules [3].

DRUG	DOXORUBICIN	DAUNORUBICIN	EPIRUBICIN
FORMULA	$C_{27}H_{29}NO_{11}$ HCl	$C_{27}H_{29}NO_{10}$ HCl	$C_{27}H_{29}NO_{11}$ HCl
MELTING POINT	177°C	205°C	173-176°C
SOLUBILITY			
WATER	SOLUBLE	SOLUBLE	SOLUBLE
CHLORO-FORM	INSOLUBLE	INSOLUBLE	INSOLUBLE
METHANOL	SOLUBLE	SOLUBLE	SOLUBLE
PARTITION COEFF. BUTANOL/ WATER	16.2	6.4	32.3
pKa	8.4	8.3	8.0
UV AND VISIBLE SPECTRA IN METHANOL λ max	234,252,290, 480,495,532nm	233,253,290, 477,495,530nm	233,253,290, 477,495,530nm

Table 1.3. Properties of doxorubicin, daunorubicin and epirubicin (from references [1,12,56]).

Key:

Solubility refers to the hydrochloride salt.
Conditions under which partition coefficient and pKa were determined not quoted in reference.

Several authors have published distribution studies of the anthracyclines in both animals and man. Unverferth et al [57] noted that doxorubicin concentrated most heavily in the kidney. Lower concentrations occurred in the liver, lung, spleen, heart, intestine and skeletal muscle. Natale et al [49] observed the lowest levels of the anthracyclines in adipose tissue and serous membranes and the highest in the gall bladder. Alberts et al [58] and Yesair [59] also noted accumulation in the heart, bone marrow, lung, spleen and white blood cells.

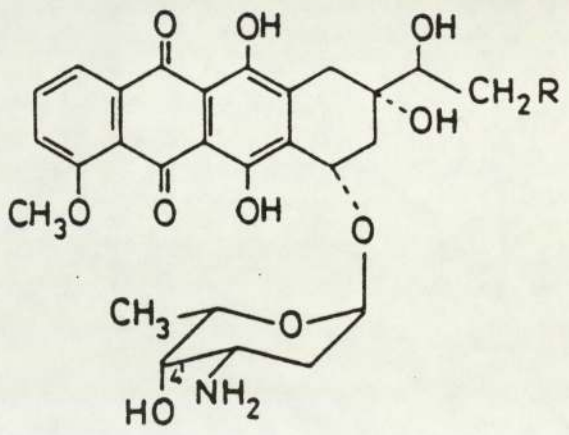
1.5.ii. Metabolism and excretion.

Available evidence indicates that doxorubicin and daunorubicin are metabolized mainly by two reactions [12]. The first involves reduction of the side chain carbonyl group to a secondary alcoholic group to form doxorubicinol and daunorubicinol [14] (Figure 1.4). This reaction is catalysed by the cytoplasmic aldoketo reductases which are found in all tissues especially the liver and kidney [14,60,61]. The second major metabolic conversion is a reductive deglycosidation, by microsomal enzymes, which results in the formation of the water-insoluble aglycones (Figure 1.5). The metabolism of daunorubicin is qualitatively similar, but more extensive, than doxorubicin since daunorubicin is a better substrate for the aldoketo reductase and microsomal enzyme systems [22,60].

The metabolism of epirubicin , in humans, differs from doxorubicin and daunorubicin. The 4-O-β-D glucuronide of both epirubicin and the 13-hydroxy metabolite, epirubicinol, are formed in considerable quantities [47,62,63,64]. This contrasts sharply with the metabolism of doxorubicin and daunorubicin where glucuronidation of the daunosamine moiety is not observed. Extensive formation of aglycones of epirubicin and epirubicinol have also been documented [47,62,63,65,67].

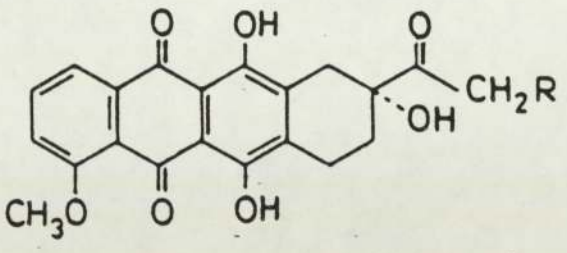
Weenen et al [63] proposed a mechanism to explain these differences in metabolism. In the doxorubicin molecule the close proximity of the 4-OH to the basic 3-NH causes a steric hindrance that prevents glucuronide formation. The glucuronidation pathway of epirubicin in man in relation to the structure of doxorubicin is shown in Figure 1.6.

The formation of significant amounts of glucuronide metabolites and the more rapid clearance of epirubicin may account for the lower observed toxicity of this analogue which has been reported in phase I and II studies [4].

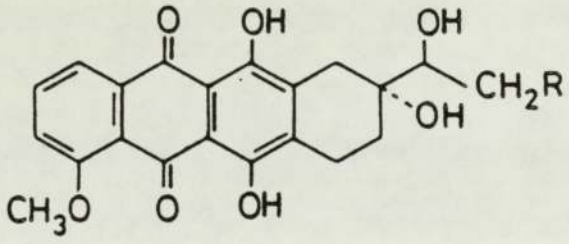


(7) doxorubicinol R = OH
(8) daunorubicinol R = H

Figure 1.4. Structure of doxorubicinol and daunorubicinol.



(9) 7-deoxydoxorubicin aglycone R = OH
(10) 7-deoxydaunorubicin aglycone R = H



(11) 7-deoxydoxorubicinol aglycone R = OH
(12) 7-deoxydaunorubicinol aglycone R = H

Figure 1.5. Structure of the 7-deoxyaglycones of doxorubicin and daunorubicin.

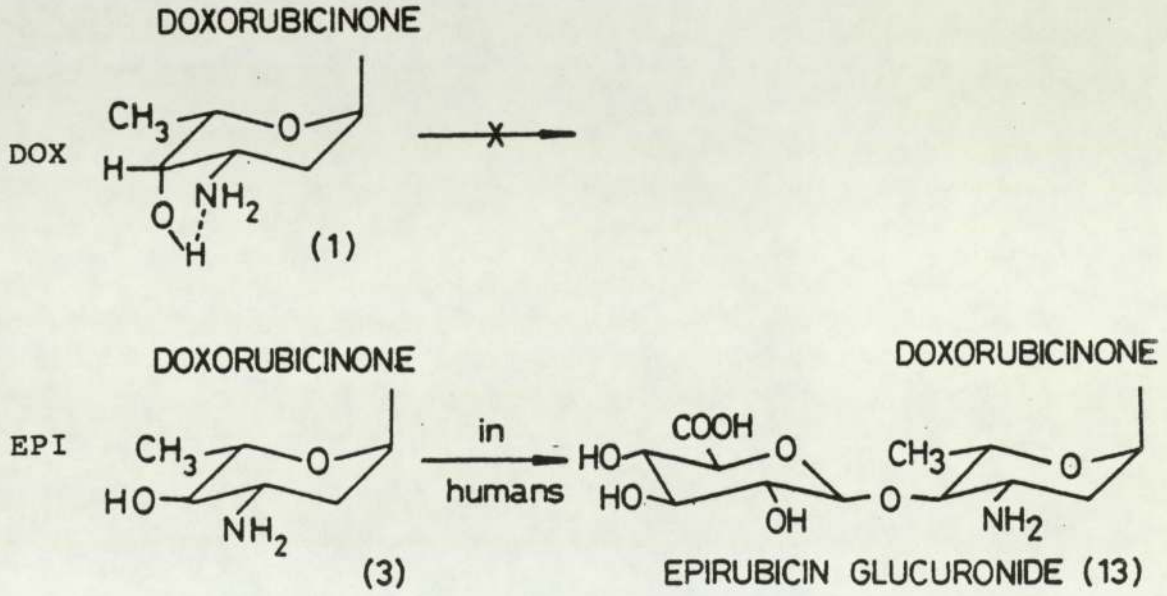


Figure 1.6. The glucuronidation pathway of epirubicin in man in relation to the structure of doxorubicin and epirubicin (from reference [66]).

Biliary excretion is the main route of elimination of daunorubicin, doxorubicin and epirubicin and their metabolites [3]. After biliary excretion the drug and metabolites are probably hydrolysed in the large intestine and excreted as aglycones in the faeces [68].

1.6. Toxicity.

The successful use of doxorubicin, daunorubicin and epirubicin has been limited by the acute toxicities common to most cancer drugs which include haematopoietic suppression, nausea, vomiting and alopecia [2,26,43,69]. The most serious side effect, which is unique to the anthracyclines, is cardiotoxicity [22,70] which may manifest itself in two ways. Firstly, acute tachycardia and ECG changes can occur within a few hours of administration. Transient ECG changes include flattening or inversion of the T wave, depression of the ST segment and arrhythmias [27]. These changes occur in an average of fifteen percent of patients [71], are generally reversible and are not an indication for cessation of treatment. If a reduction in the QRS voltage is observed the benefit of continuation of treatment versus the possibility of cardiac damage must be assessed [27].

The second manifestation of cardiac toxicity is chronic cardiomyopathy which for doxorubicin may become apparent days, weeks or even years after the last administration of the drug [72]. The resultant irreversible and often fatal

(60% of cases) congestive heart failure (CHF) [73,74] develops suddenly. Classical signs of CHF include; tachycardia, tachypnoea, hepatomegaly, cardiomegaly, peripheral or pulmonary oedema, venous congestion and pleural effusion [75,76]. Cardiac failure may also occur without any prior ECG changes [27]. Histological examination of the heart shows an initially focal swelling of the sarcoplasmic reticulum and myofibrillar dropout which eventually, as damage progresses, becomes diffuse [2].

The occurrence of cardiotoxicity is proportional to the total dose administered [43,46,72,73,77,78,79]. In order to limit the incidence of this potentially fatal side effect to within clinically acceptable levels the maximum cumulative dose of doxorubicin, daunorubicin and epirubicin is limited to 450-550mg/m^2 ,500mg/m^2 and 700mg/m^2 respectively.

Use of an empirical upper dose limit for doxorubicin has its disadvantages since individual sensitivity to the drug varies greatly [57,80]. Although cardiac monitoring can reduce the risk of cardiotoxicity [57,81] the ideal solution would be to identify and negate the cause of this toxic effect. Great effort has been expended to discover a means of ameliorating, preventing, or at least delaying, the onset of cardiotoxicity. Some of the areas under investigation include detailed studies of risk factors, synthesis of analogues, investigation of the pathogenesis of the cardiomyopathy, evaluation of potential antidotes and dosage schedule alteration.

1.6.i. Detailed studies of risk factors.

The most important determinant for the development of congestive heart failure is the total dose administered [57,75]. Figure 1.7 shows the probability of developing doxorubicin-induced CHF versus the total cumulative dose calculated by Von Hoff *et al* [77]. The second most important factor is prior or concurrent mediastinal irradiation [70,71,73,77,81,82,83] especially at doses greater than two thousand rads [81]. Many investigators have indicated that patients over 70 years old have poorer cardiac assessment scores [84,85] and an increased risk of developing CHF [70,77,82,86]. Figure 1.8 shows the probability of developing doxorubicin-induced CHF for patients in four arbitrary age categories (from Von Hoff *et al* [77]). Concurrent cyclophosphamide therapy also appears to potentiate cardiac damage [71,73,87]. For this reason the manufacturers recommend dosage reduction of the anthracycline if used in combination with cyclophosphamide (section 1.3). Several authors have noted an association between pre-treatment cardiac disease and hypertension with a greater risk of development of cardiomyopathy [71,73,77,82]. Figure 1.9 shows the probability of developing doxorubicin-induced CHF for patients with and without previous cardiac disease (from Von Hoff *et al* [77]). This association has not been well studied because patients with these risk factors do not usually receive anthracycline therapy.

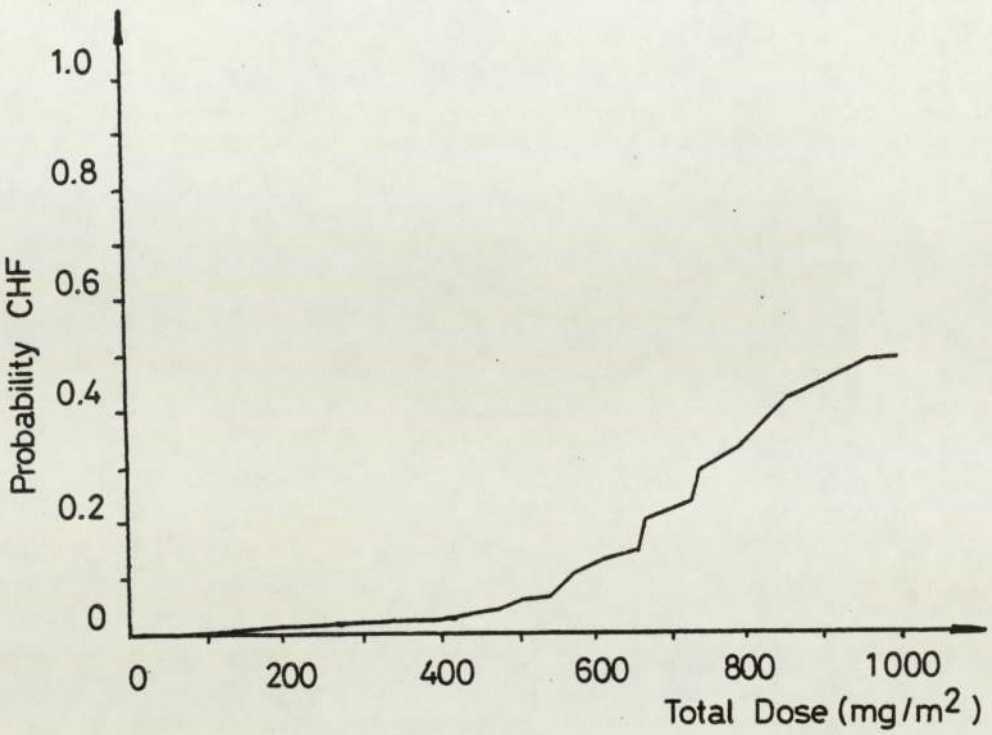


Figure 1.7. Cumulative probability of developing doxorubicin-induced congestive heart failure (CHF) versus total cumulative dose of doxorubicin (from Von Hoff et al [77]).

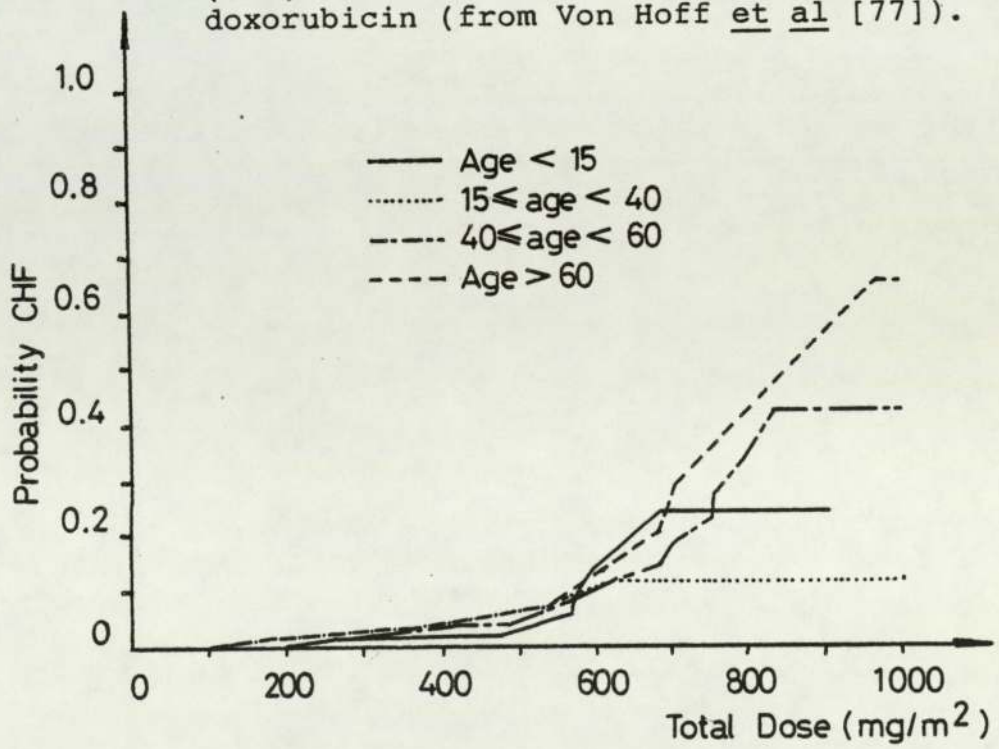


Figure 1.8. Cumulative probability of developing doxorubicin-induced CHF versus total cumulative dose of doxorubicin for patients in four arbitrary age categories (from Von Hoff et al [77]).

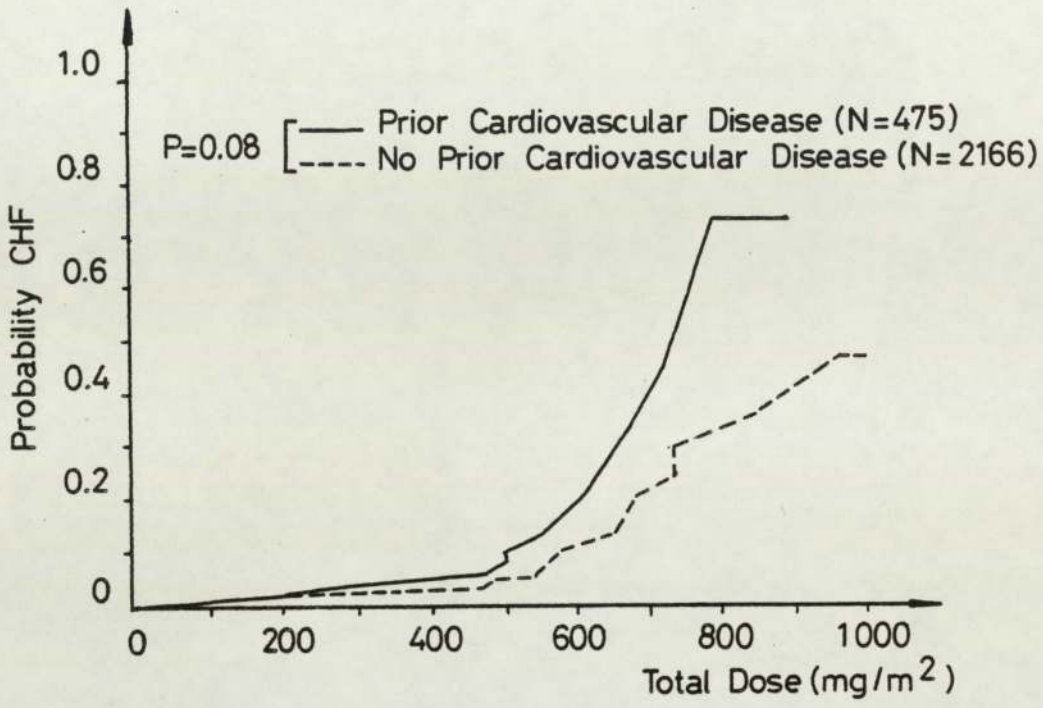


Figure 1.9. Cumulative probability of developing doxorubicin-induced CHF versus total cumulative dose of doxorubicin for patients with and without previous cardiovascular disease (from Von Hoff et al [77]).

1.6.ii. Synthesis of analogues.

Anthracycline analogues have been obtained from fungi isolated from soil samples and by rational synthesis. Many modifications have involved the C9 side chain as substitution at this site is easy to accomplish chemically. Another major site for modifications has been the aminosugar side chain at C7. The third major area of structural modification has been the anthraquinone rings. Ring D has been the site of most modifications because of the simplicity of such structural modifications in the rational synthesis of the parent compound [3].

The clinical success of doxorubicin, which resulted from a minor structural change, encouraged the search for other anthracycline analogues. Creation of analogues with less acute toxicity and/or less cardiac toxicity was of particular interest. New developments also needed to encompass an enlargement of the antitumour spectrum for unresponsive tumours such as non-small cell lung cancer, pancreatic and colorectal carcinomas and melanoma [1,88,89]. An increase in response rate of tumours that were already sensitive was also desirable [88].

Over one thousand anthracycline analogues have been synthesized but only a few have undergone clinical testing. The most extensively developed analogue is epirubicin. Epirubicin was synthesized at Farmitalia in an attempt to improve treatment by decreasing cardiotoxicity and widening the range of neoplasms against which the anthracyclines were active. Pre-clinical studies showed that epirubicin had an equal antitumour activity and was less cardiotoxic than doxorubicin in several animal species [90,91]. For these reasons epirubicin was selected for further development. In phase I studies epirubicin showed fewer episodes of nausea and vomiting and fewer patients developed marked alopecia. In phase II studies epirubicin showed antitumour activity in a wide range of neoplasms [89] (section 1.3).

The increase in therapeutic index for epirubicin suggested in the preclinical studies has been partially validated in clinical trials. Epirubicin has a spectrum of activity qualitatively and quantitatively similar to

doxorubicin. It is less certain that there is an advantage in reduced acute toxicity when the two agents are given in equally myelosuppressive doses [3]. The efficacy of epirubicin as a single agent and in combination regimes, in breast cancer, is currently being compared to responses obtained for doxorubicin (section 1.3).

Bargiotti et al [92] synthesized the 4'C-methyl and 4'C-methyl-4'-O-methyl analogues of daunorubicin and doxorubicin. The most active of these compounds against the P388 leukaemia was the 4'O-methyl doxorubicin analogue which is shown in Figure 1.10. This derivative was also found to be less cardiotoxic and more active than its parent compound against L1210 leukaemia [93] and colon adenocarcinomas [94]. Further studies are being conducted with this derivative in experimental models.

Esorubicin, (4-deoxydoxorubicin) is an analogue in which the hydroxyl group is deleted from the C4' position [87]. Esorubicin is otherwise structurally identical to doxorubicin. The structure of 4-deoxydoxorubicin is shown in Figure 1.11. Initial tumour testing indicated possible advantages over doxorubicin. Esorubicin produced less alopecia and vomiting than doxorubicin. The frequency and severity of cardiotoxicity has not been fully determined. Unless esorubicin shows antitumour efficacy which is at least equivalent to doxorubicin this compound will not be studied further [3].

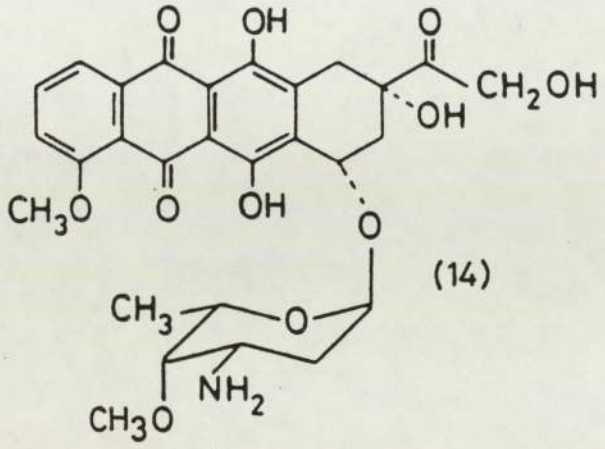


Figure 1.10. Structure of 4-0-methyldoxorubicin.

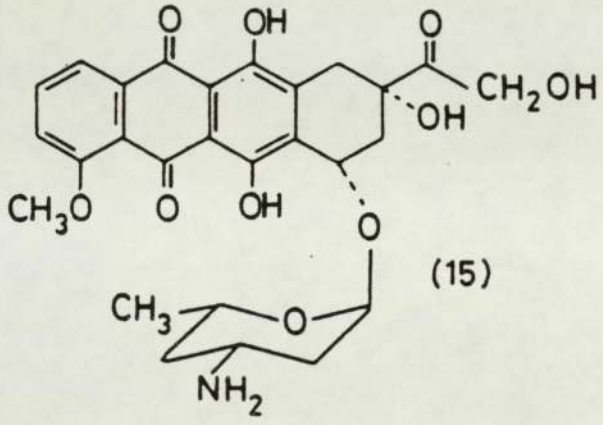


Figure 1.11. Structure of 4-deoxydoxorubicin.

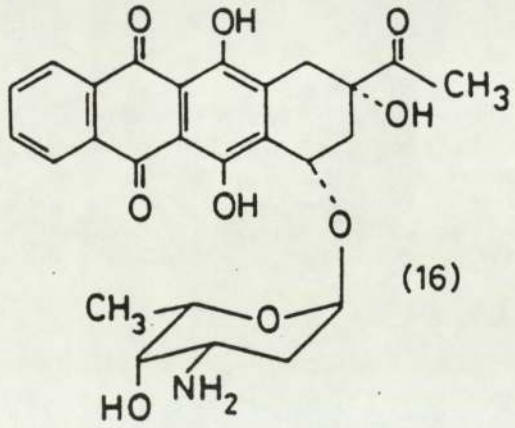


Figure 1.12. Structure of 4-demethoxydaunorubicin.

Idarubicin (4-demethoxydaunorubicin) is an analogue in which the methoxy group at C4 in ring D has been removed [88]. The structure of 4-demethoxydaunorubicin is shown in Figure 1.12. The lack of the methoxy group is the only structural difference between idarubicin and daunorubicin. The therapeutic advantage of idarubicin over daunorubicin and differences in toxicity appear to be marginal. One advantage is that idarubicin may be administered orally. At present, too few patients have been treated with high cumulative doses of idarubicin to assess cardiotoxicity [3].

1.6.iii. Investigation of the pathogenesis of the cardiomyopathy.

The ability of anthracyclines to trigger formation of oxygen radicals was first noted by Sato et al [95]. Those authors demonstrated that microsomal enzyme P450 reductase was able to catalyze the reduction of doxorubicin or daunorubicin to a semiquinone free radical. The semiquinone in turn rapidly reduced molecular oxygen to the superoxide ion. Myers et al [96] observed that a free radical scavenger lessened cardiac damage resulting from doxorubicin administration. This led to the proposal that oxygen-radical-mediated membrane damage in cardiac tissue was the mechanism of cardiomyopathy. Free radicals cause cell membrane damage through peroxidation of lipids [96,97]. The explanation for the selective damage of cardiac cells centres around mitochondria which are abundant in cardiac

tissue. Mitochondria are known sites of superoxide production [98,99]. In addition cardiac mitochondria have an unusually active electron transport chain. Doxorubicin-mediated oxygen radical formation occurs in the initial stages of mitochondrial electron transport. Figure 1.13 shows the mechanism for generation of free radicals inside mitochondria proposed by Milei et al [100]. It is possible that cardiac mitochondria are a particularly rich source of doxorubicin generated superoxide and hydrogen peroxide [2].

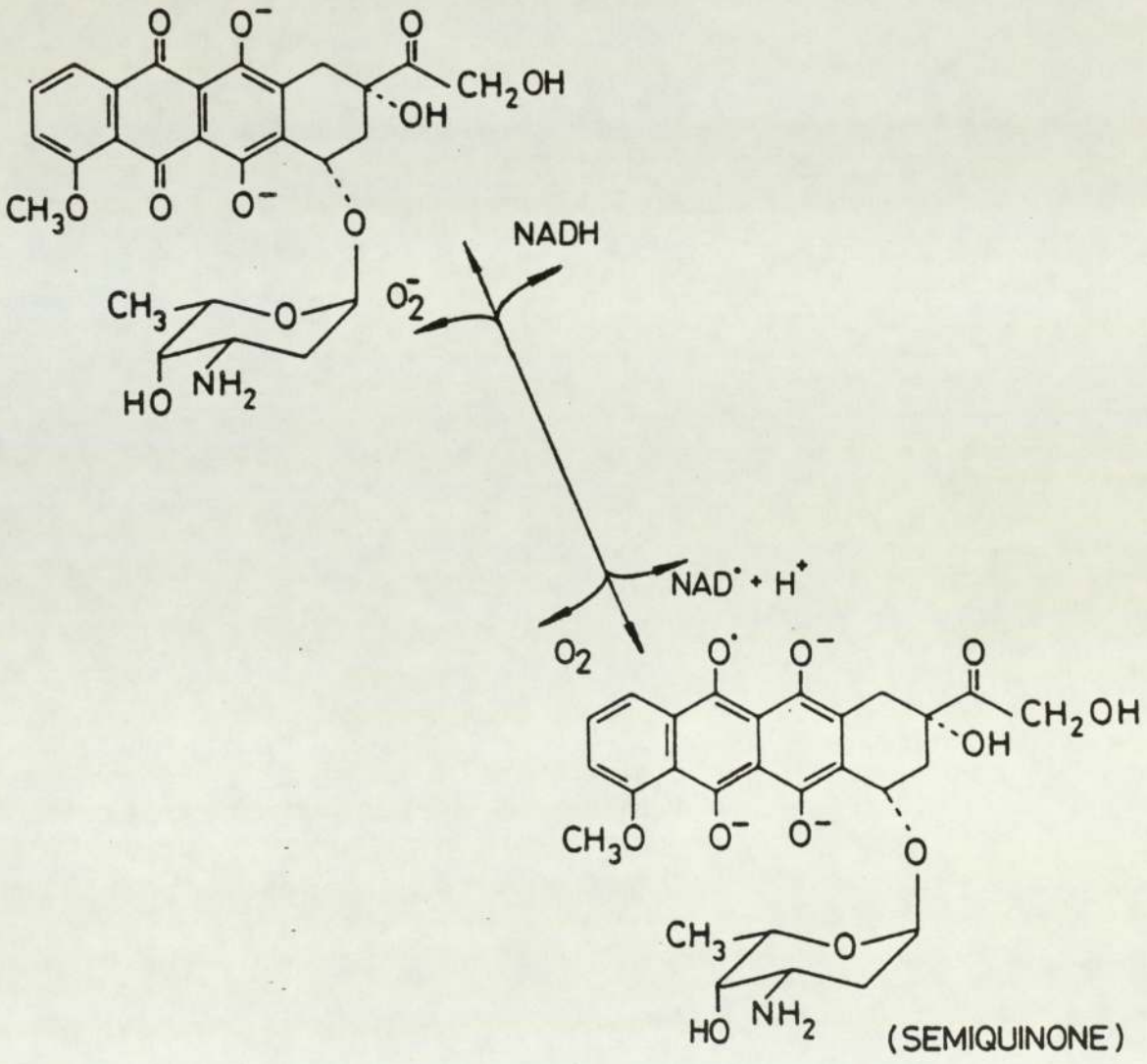


Figure 1.13. Generation of free radicals inside mitochondria (from Milei et al [100]).

A second cause of tissue selectivity is related to the way in which cardiac tissue defends itself against oxygen radical damage. In general, mammalian cells detoxify oxygen radicals by first converting superoxide to hydrogen peroxide by the enzymatic action of superoxide dismutase [2]. Elimination of the hydrogen peroxide is afforded by either catalase or glutathione peroxidase [2]. Cardiac detoxification appears to rely upon superoxide dismutase and glutathione peroxidase [101]. Doxorubicin administration results in a rapid drop in the levels of glutathione peroxidase followed by a gradual recovery over a period of three or four days [2]. In summary, it appears that doxorubicin can stimulate free radical production and disrupt a key step in the detoxification process of these species. The net result of these effects is the onset of cardiac damage.

The second hypothesis which has been forwarded to explain certain aspects of the cardiac toxicity of the anthracyclines is a direct membrane effect. Doxorubicin binds to a variety of membrane sites including spectrin and cardiolipin [102]. Increased cardiolipin content in membranes appears to be a shared characteristic of malignant cells and cardiac mitochondria [103]. This binding has been seen to cause alterations in membrane structure and ion transport mechanisms.

A third hypothesis which has been put forward as a mechanism for doxorubicin-induced cardiotoxicity is through an increase in intra-cellular calcium concentrations and changes in calcium ion handling in the myocardium [75]

which have been associated with doxorubicin-induced cardiomyopathy in animals [2]. However, it is doubtful that these changes are linked to the chronic cardiomyopathy seen with the anthracyclines in humans [75]. The role of calcium ion transport in acute cardiotoxicity remains to be established [75].

1.6.iv. Evaluation of potential antidotes.

A variety of drugs have been used to prevent the occurrence of doxorubicin-induced cardiomyopathy. The free radical scavengers alpha-tocopherol (vitamin E) [97,100,104,105] and N-acetylcysteine [106,107] have been extensively studied. These agents have produced some encouraging results in animal studies but doses and regimes varied considerably and may be inappropriate for human use [75].

Guthrie [108] observed that patients receiving digitalis glycosides at the time of doxorubicin administration seemed to have less cardiac damage. In another study Whittaker and Ismail [104] randomly assigned patients to control groups or to receive either digoxin or vitamin E. Results suggested that digoxin may protect the heart against doxorubicin damage. No significant difference was found between the control and vitamin E groups.

Selenium is a co-factor for cardiac glutathione peroxidase and plays a role in the detoxification of free radicals. The cardioprotective effect of selenium has been assessed alone and in combination with vitamin E. When used

in combination with vitamin E a decrease in doxorubicin cardiomyopathy was noted in the rabbit [109,110] but not in the dog [111] or the pig [112] or the mouse [105]. Selenium alone appeared to be of no benefit in rabbits [109]. It appears unlikely that selenium will prove beneficial in the prophylaxis of doxorubicin-induced cardiomyopathy.

Carnitine is a naturally occurring quaternary ammonium compound, found in relatively high quantities in cardiac tissue. Carnitine plays a critical role in heart metabolism and is required for transmembranous transport of long chain fatty acids into mitochondria [113]. Carnitine also helps regulate acetyl CoA levels through the enzyme carnitine acetyltransferase [114]. Several authors studies have shown that carnitine deficiency is associated with cardiomyopathy [115-118]. Studies which have attempted to show a protective effect of L-carnitine against doxorubicin-induced cardiomyopathy have produced both positive and negative results [119-121]. Mc Falls et al [119] induced cardiomyopathy in rats over a six to seven week period by weekly injections of doxorubicin, 2mg/kg. Treatment with intra-peritoneal (IP) L-carnitine (500mg/kg/day) reduced histopathological alterations in cardiac tissue and improved cardiac performance. Alberts et al [120] studied the effect of DL-carnitine on acute and chronic cardiomyopathy in mice. In this study carnitine was given IP in a dose of 200mg/kg for 3 days followed by indefinite maintenance therapy of 2mg of carnitine per ml of drinking water. Results showed that carnitine treatment significantly

decreased both acute high-dose and chronic, intermittent, low-dose adriamycin toxicity in normal mice. Goldsmith et al investigated doxorubicin-induced cardiomyopathy in rabbits. Results from this study showed that carnitine given in a dose of 180mg/kg intravenously on five days of each week failed to prevent cardiomyopathy.

The differences in the results obtained by these authors may be partially explained by inherent differences in the animal models and/or differences in the doses and schedule of carnitine used by the investigators. In addition, Alberts et al [120] used DL-carnitine in their study and Mc Falls et al [119] used the L isomer whereas Goldsmith et al [121] did not specify which isomer was used in their study. As the L isomer is the physiologically active form [113] this may also account for the divergent results in these studies.

In summary, conflicting results have been obtained with these and other agents in both animal models and man. Useful cardioprotective agents for human medication have not, as yet, been demonstrated. Use of antidotes to doxorubicin-induced cardiomyopathy is probably the least successful attempt to alleviate cardiac damage produced by the anthracyclines.

1.6.v. Dosage schedule alteration.

The standard dosage schedule for doxorubicin chemotherapy is intermittent high dose bolus treatment (60-75mg/m^2) repeated at three-weekly intervals (section 1.3).

This regime allows practical and convenient ambulatory treatment and permits immunologic and haematologic parameters to recover [122].

A schedule of weekly low dose doxorubicin has been claimed to be associated with a lower frequency of side effects, including less cardiotoxicity [43,78,123,124] but with retention of antineoplastic activity [25,77,123-130]. This permits an extended duration of therapy and an increase in the maximum cumulative dose [122,131].

Several studies have suggested that constant or protracted infusions might have some advantages over intravenous bolus therapy [26,78,132] by improvement of the therapeutic index and tumour kill. Prolonged and continuous infusion of doxorubicin have also been claimed to be even more advantageous than the weekly low dose schedule, causing no more, or even fewer, side effects [26,43,78,125,130, 133-135].

The cytostatic effect of doxorubicin <u>in vitro</u> and <u>in vivo</u> has been correlated with the area under the plasma concentration-time curve (AUC) [69,136]. Side effects have been correlated with either the AUC (haematopoietic effects) or the maximum plasma concentration (Cp max) (cardiotoxicity, nausea and vomiting). By dose fractionation and prolonged infusion it is possible to decrease Cp max and therefore cardiotoxicity, nausea and vomiting [78,122,131, 132,133], but maintain the AUC and therefore the therapeutic effect [43]. Dosage schedule alteration has been one of the most successful attempts to increase the therapeutic index and decrease some of the side effects of doxorubicin [43].

1.7. Methods of analysis.

The perfect assay method must be highly sensitive (in the nanomolar range), have adequate specificity and be able to quantify the drugs being analysed with a high level of reproducibility. A number of analytical approaches have been described for the estimation of the anthracyclines in biological fluids. These methods all suffer from one or more disadvantages which are detailed below.

1.7.i. Non-chromatographic procedures.

Initially, the anthracyclines were quantified by fluorimetric assays. Rosso et al [137] used a method previously reported for the assay of daunorubicin by Finkel et al [138] which involved direct fluorimetric determination of an n-butanol extract of plasma or urine. Direct radioimmunoassay (RIA) procedures for the determination of the anthracyclines have also been reported in the literature [139-141].

Both fluorimetry and RIA showed a very high sensitivity and quantitative detection in the region of low ng/ml in plasma. Both methods were useful for the quantitation of the total anthracycline concentration but neither method differentiated between parent drug and metabolites [46].

1.7.ii. Chromatographic procedures.

Thin-layer chromatography (TLC) can be a labour intensive and a tedious process [46,142] but has been the most widely used technique for both quantitative and qualitative analysis of the anthracyclines. The anthracycline chromophore is both light and air sensitive. Chromophore bleaching prior to fluorescent analysis can lead to under estimation of the drugs. The acidic nature of the silica gel can also cause hydrolytic cleavage of the glycosidic bond which results in the formation of aglycone artefacts [46,142,143]. Israel et al [143] also noted that artefact production was even more pronounced when the plates were developed in acetic acid-containing solvent mixtures. In a comparison of TLC and HPLC Brenner et al [142] observed that synthesis and co-chromatography of known aglycone metabolites with the plasma specimens improved the accuracy of TLC analysis, but concluded that HPLC was the preferred technique.

The HPLC methods in the literature can easily be adapted to assay almost all the anthracyclines [144]. Excellent separation of parent drugs and metabolites can be obtained by both normal-phase [143,145,146] and reversed-phase systems [62,143,147-152]. One advantage of normal-phase systems is that organic extracts can be injected directly onto the chromatographic system [153]. The major drawback of normal-phase systems is their inability to separate aglycones [154,155]. Israel et al [143] concluded

that reversed-phase systems gave a more rapid and a clearer separation than normal-phase systems which often exhibited peak tailing. Aglycones and conjugate metabolites can be simultaneously determined in biological samples depending on the work-up procedure [143,152,154,156]. Gradient systems [62,152] are necessary only for complete characterization of intact drugs and metabolites.

Langone et al [156] described a method in which reversed-phase HPLC was used to separate doxorubicin from doxorubicinol and aglycones in urine and RIA used as the method of quantitation. This combination offered a highly efficient, sensitive and simple procedure but the methods were lengthy and tedious. HPLC separation required thirty minutes. Finally, lyophillization of up to thirty fractions was required before quantitation by RIA.

The method of detection depends upon the type and concentration of the sample. Sensitive and selective detection (2 - 5ng/ml) can be obtained using a fluorescence detector with the excitation wavelength set at approximately 480nm and fluorescence measured in the 500-600nm range. Ultraviolet (UV) detection is two or three times less sensitive than fluorescence detection [157].

In conclusion, sample handling and extraction procedures must be carried out by methods which avoid artefact production prior to quantitative determination of the anthracyclines and their metabolites in biological samples. The most convenient, sensitive, specific method of analysis for the anthracyclines is reversed-phase HPLC.

1.8.Stability.

1.8.i. Degradation in acidic and alkaline media.

Decomposition of doxorubicin and daunorubicin in acidic solution has been studied by several authors [158,159]. Acidic hydrolysis of doxorubicin, daunorubicin and epirubicin yields a red-coloured, water-insoluble aglycone and a water-soluble aminosugar [160] (Figure 1.14). Linear regression analysis of a plot of the natural logarithm of the residual doxorubicin concentration versus time showed that degradation (pH 0.4 to 2.1) followed first order kinetics [158].

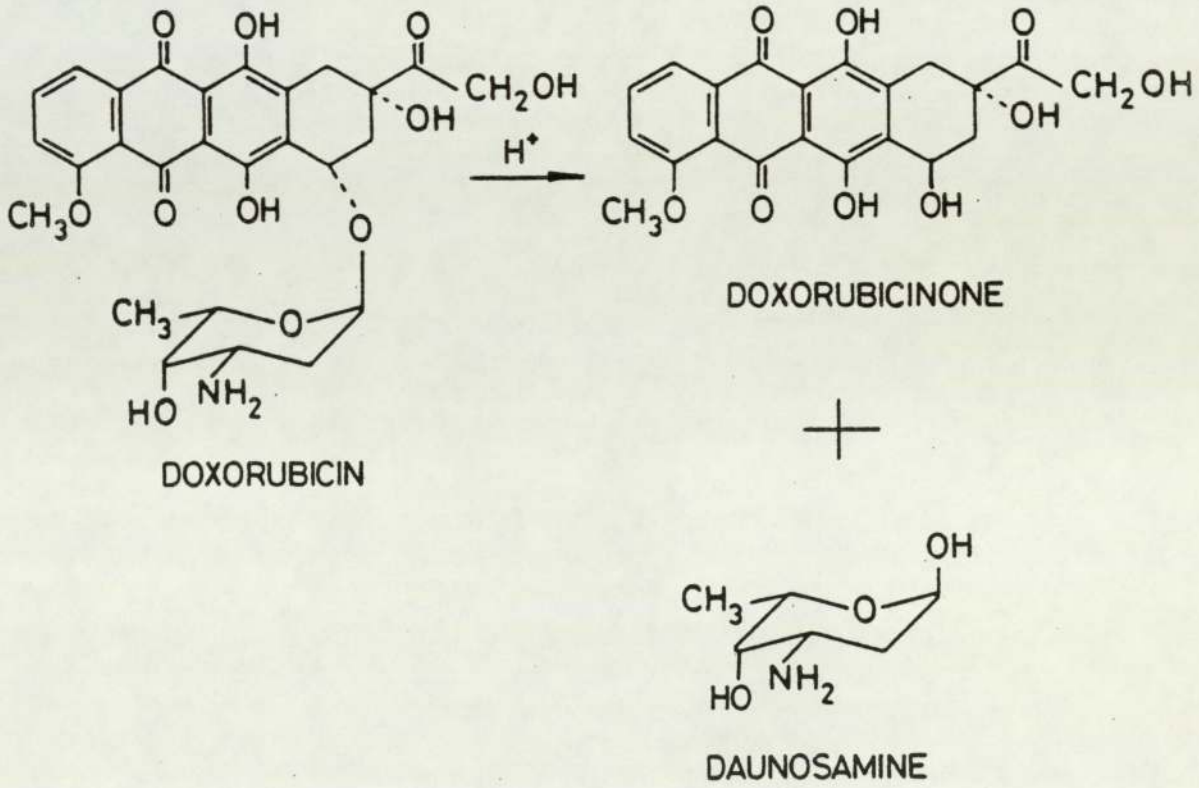


Figure 1.14. Degradation of doxorubicin in acidic solution.

On addition to strongly alkaline solution a colour change from red to deep purple occurs immediately. Cleavage of the amino sugar is thought to occur which results in an ineffective moiety [160]. Several studies have noted the presence of degradation products in alkaline media [150,161,162] which were not identified except in the study of Abdeen [163].

A more detailed account of the degradation of daunorubicin and doxorubicin in acidic solution has recently been published by Beijnen et al [184]. These authors have subsequently identified the degradation products of daunorubicin in acidic and alkaline solution. As these data were published after results in the following experiments were obtained they will be fully discussed in the appropriate chapter.

1.8.ii.Photodegradation.

Di Marco [164] reported that daunorubicin formed a stable complex with DNA under ultraviolet (UV) activation. Subsequently photoactivation studies have been carried out by several authors [165-167]. Those investigators showed that doxorubicin and daunorubicin formed covalent bonds to DNA. The drug-DNA binding was found to be catalysed not only by UV irradiation but by visible light at 470nm [168].

Isolation of products of the photoactivation DNA reaction yielded predominantly aglycones, deoxyaglycone structures, with loss of the 9-position acetyl group, and drug polymers. UV irradiation of daunorubicin also produced

hydrogen peroxide which indicated that oxygen was interacting with the anthracycline structure and undergoing redox reactions. These redox reactions were presumed to occur through homolytic type reactions with production of free radical intermediates.

Tavoloni et al [169] investigated the kinetics of degradation of doxorubicin in several solvents. Studies were completed in the dark, in room and intense light, between 500µg/ml and 10µg/ml. All drug concentrations were determined fluorimetrically [170].

The stability of doxorubicin in the dark was found to be independent of drug concentration over the range 10µg/ml to 500µg/ml. No degradation was observed in the dark irrespective of the solvent used. The rate of photodegradation was directly proportional to the strength of light used (10 to 500µg/ml) and inversely proportional to the drug concentration (10 to 500µg/ml). Above 100µg/ml in room light little or no photodegradation was observed [169].

The amount of photodegradation of doxorubicin also appeared to be affected by the nature of the solvent used. Doxorubicin was observed to be most stable in bile and progressively less stable in ethanol, sodium chloride 0.9%, distilled water and Ringer-Krebs bicarbonate. The authors postulated that the yellow-green colour of bile or chemical or physicochemical interaction between the drug and solvent may have protected against photodegradation. Daugherty and Hixon [171] postulated that the protective effect may have

been partially due to the presence of butylated hydroxytoluene, its metabolites or another free radical scavenger in the bile. Conversely, Gutteridge and Wilkins [168] concluded that the addition of oxygen radical scavengers did not prevent photodegradation. Tavoloni et al [169] offered no explanation for the recorded differences in stability observed in ethanol, sodium chloride 0.9%, distilled water or Ringer-Krebs bicarbonate.

Data were evaluated for first order kinetics. Plots of the natural logarithm of the concentration remaining versus time were linear and indicated that photodegradation of doxorubicin followed first order kinetics [169].

1.8.iii. Stability in infusion fluids.

Information on the stability of doxorubicin is available in the literature [172-177] but the data are limited and contradictory. The stability of the anthracyclines is affected by many factors such as the pH of the medium and the storage temperature but one of the most important of these factors is photodegradation. Many of the stability studies in the literature, some of which are shown in Table 1.4, do not state whether the solutions were protected from light. Others have examined degradation under fluorescent light [173]. The discrepancy of the literature data, concerning the stability of doxorubicin in infusion fluids, may be due to a lack of control of photodegradation or to differences in the pH of the infusion fluids studied, or both.

TEMP. (°C)	CONC. (µg/ml)	pH	DILUENT	LIGHT	LENGTH OF EXPERIMENT/ STABILITY	ASSAY	REF.
-20	1.5	?	NS	?DARK	21d/STABLE	BA	178
-20	0.6	?	NS	?DARK	21d/UNSTABLE	BA	178
-20	1400	?	NS	?DARK	30d/STABLE	HPLC	174
-20	2000	?	WATER	?DARK	30d/UNSTABLE	HPLC	177
-20	1000	4.8	NS	?DARK	14d/STABLE	HPLC	175
4	2000	?	WATER	?DARK	180d/UNSTABLE	HPLC	177
4	1.5	?	NS	?DARK	21d/STABLE	BA	178
4	0.6	?	NS	?DARK	21d/UNSTABLE	BA	178
21	10/20	4.5	DEX.5%	RL	72hr/STABLE	HPLC	173
21	10/20	6.2	NS	RL	72hr/UNSTABLE	HPLC	173
25	100	4.7	DEX.5%	DARK	28d/STABLE	HPLC	176
25	100	4.4	DEX/SAL	DARK	28d/STABLE	HPLC	176
25	100	7.0	NS	DARK	28d/UNSTABLE	HPLC	176
RT	180	4.2	DEX.5%	RL	48hr/STABLE	HPLC	172
RT	180	4.75	DEX.5%	RL	40hr/UNSTABLE	HPLC	172

Table 1.4. Stability data for doxorubicin.

Key: NS= sodium chloride 0.9%, RL= room light,
BA= biological assay,
HPLC= high performance liquid chromatography.

Poochikian et al [173] studied the stability of doxorubicin and daunorubicin in dextrose, pH 4.5, and sodium chloride, pH 6.2, in glass containers under fluorescent light. Results showed that doxorubicin and daunorubicin became progressively more stable as the pH of the drug infusion fluid admixture became more acidic with optimum stability in dextrose 5%. Data obtained by Tavoloni et al [169] under virtually identical conditions showed the solutions to be markedly less stable.

Benvenuto et al [172] studied the stability and compatibility of doxorubicin in glass and plastic containers which were stored at room temperature and were not protected from light. Results showed that doxorubicin was more stable in dextrose 5% in plastic than in glass. The decrease in concentration of doxorubicin was accompanied by a colour change from clear orange to clear reddish orange and a pH increase from 4.75 to 6.26. Tabulated data in this reference showed pH values for the drug in sodium chloride 0.9% and quoted stability data for dextrose 5% which was rather misleading.

Beijnen et al [176], in a study carried out under more controlled conditions (25°C in the dark), did not observe a pH increase in any of the infusion fluids studied. Conversely, the pH of the sodium chloride 0.9% remained the same and the pH of the dextrose-containing solutions decreased by approximately one unit. These authors observed that, over a four week period, doxorubicin and epirubicin were stable in dextrose-containing infusion fluids at pH

4.4 and 4.7 but unstable in sodium chloride 0.9% at pH 7.0 where degradation was accompanied by formation of a red-coloured precipitate. Daunorubicin was found to be stable in both dextrose and sodium chloride-containing infusion fluids.

Beijnen et al [176] concluded that structural variations in the 4'-position of the amino sugar moiety did not influence chemical stability in the infusion fluids studied. Stability in sodium chloride 0.9% appeared to be affected by the substituent on the C14 atom in the aglycone portion of the molecule.

Several authors have published data on the effects of freezing and thawing the anthracyclines. Hoffman et al [177] observed that frozen samples of an aqueous solution of doxorubicin (2mg/ml), in glass vials, could be stored at -20° C for one month without significant degradation. These authors also stated that solutions reconstituted with sodium chloride should not be frozen. Other authors observed that doxorubicin could be frozen in sodium chloride 0.9% at 1.4mg/ml for thirty days [174] or at 100µg/ml for ten weeks [179]. The effect of thawing doxorubicin [174,175] and epirubicin [175] frozen in sodium chloride 0.9%, in PVC containers, by microwave radiation has also been investigated. After four re-thawings in a microwave a decrease in doxorubicin concentration was noted by Karlsen et al [174]. Conversely, Hoffman et al [177] observed that doxorubicin in aqueous solution could be re-thawed seven times at room temperature without significant loss in potency. Uneven distribution of microwave energy may

overheat solutions and lead to degradation. This problem may explain the observed differences of the effects of re-thawing observed by these authors.

1.9. Conclusion.

Many of the results obtained in stability studies with doxorubicin have produced divergent results. Large differences in stability have been found by different groups for virtually identical experiments [169,173]. Reported storage conditions varied and in some experiments photodegradation was not controlled. Some authors used biological assay for stability assessment. Stability-indicating assays (those which have been proved to detect parent drug unequivocally are, almost by definition, not biological) are the only way to obtain reliable data. For this reason stability-indicating HPLC assays were developed for doxorubicin, daunorubicin and epirubicin and used for drug quantification in all experimental work.

Photodegradation of doxorubicin has been quantified by fluorimetric assay. Tavoloni et al [169] stated that photodegradation resulted in breakdown of the naphthacenequinone nucleus. Conversely, other authors have indicated that aglycones may be formed as photodegradation products [164]. Fluorescence does not differentiate between parent drug and metabolites [46]. If aglycones were formed as photodegradation products the rates of degradation presented by these authors may be inaccurate. There is no published data which has compared the photodegradation of

doxorubicin, daunorubicin and epirubicin using HPLC assay. For this reason the rate of photodegradation of these analogues, in glass and plastic containers, under fluorescent light was compared to degradation in control solutions stored in the dark. Photodegradation may be accelerated in alkaline solution. To examine this effect the photodegradation of doxorubicin in alkaline buffer was compared with unbuffered aqueous solutions.

Degradation of doxorubicin, daunorubicin and epirubicin in acidic solution has been studied (section 1.8.i.). Until recently no data have been published on the degradation of these analogues in alkaline solution. For this reason a pH-profile was constructed for each drug.

Finally, the results obtained in these preliminary experiments were applied to the clinical situation. There are no data in the literature which have comprehensively compared the stability of these analogues in infusion fluids. If long term infusions are used, or if solutions are stored for prolonged periods before use, a strictly controlled study is required to compare the stability of these agents. Stability in plastic and glass containers was investigated. The effects of the storage temperature, the pH and type of solvent used were studied. To clarify variable results in the literature, repeated freezing and thawing of solutions was investigated. Results obtained were critically compared to those already published in order to more clearly define the stability profiles of these drugs.

Chapter Two

Development of HPLC assays for doxorubicin, daunorubicin and epirubicin.

2.1. Materials and equipment.

Unless otherwise stated the following chemicals were used throughout the experimental work: Doxorubicin, epirubicin and daunorubicin were purchased as the commercially available pharmaceutical dosage forms Adriamycin, Pharmorubicin (both Farmitalia) and Cerubicin (May and Baker) respectively as specified in Table 1.1. All methanol and acetonitrile was HPLC grade (Fisons Scientific, Loughborough). Orthophosphoric acid, all other solvents and chemicals were AnalaR grade (BDH) and were used without further purification. Water was deionized prior to use.

The HPLC system consisted of an Altex 100A pump which was used to deliver eluent to a 10cm x 4.6mm stainless steel Shandon column packed with ODS Hypersil 5µm reversed-phase material. Injections were made with a Rheodyne model 7125 sample injector equipped with a 10µl injection loop. A Pye-Unicam LC-UV variable wavelength detector, equipped with an 8µl flow cell, was operated at 290nm and connected to a JJ Instruments CR452 chart recorder.

2.2. Selection of mobile phase.

The aim of this work was to develop specific and reproducible stability-indicating HPLC assays for doxorubicin, daunorubicin and epirubicin. Assays were

designed to separate parent compounds from their respective degradation products in acidic and alkaline media as rapidly as possible. A reversed-phase HPLC system, with UV detection, was chosen (section 1.7.ii.) and used in all experimental work.

The final choice of assay conditions was made after assessment of several mobile phases. Initial studies investigated an eluent which contained equal parts of acetonitrile and phosphate buffer (0.015M, pH 4.6). Extensive bubble formation, which persisted even after prolonged sonication, was a problem with this mobile phase. For this reason, eluents which contained simple acetonitrile:water combinations were investigated. A mobile phase used by Beijnen et al [159] which consisted of acetonitrile:water (50:50 vol:vol) adjusted to pH 2.0 with perchloric acid was chosen as a base on which to develop the assays used in the following work. Perchloric acid was considered to be particularly hazardous and for this reason 10% orthophosphoric acid (vol:vol) was substituted for pH adjustment.

Assays were developed for each anthracycline by systematic adjustment of the percentage acetonitrile and the pH of the eluent until the desired separation was produced. Addition of ten drops of diethylamine to each litre of eluent, prior to pH adjustment, reduced peak tailing and increased peak sharpness.

The stability-indicating capacity of these methods was demonstrated by comparison of a standard solution and a fully degraded solution. Decomposition was accelerated by

subjecting the solutions to extremes of temperature and pH. Fully alkali-degraded solutions were prepared by mixing equal volumes of drug solution and Britton-Robinson buffer, pH 11.98, (Appendix I) for three hours (doxorubicin and epirubicin) or twenty four hours (daunorubicin). Fully acid-degraded solutions were prepared by mixing equal volumes of drug solution with Sorenson buffer, pH 1.4, (Appendix II) and heating in a water bath, at 80°C, for forty minutes (daunorubicin and doxorubicin) or three hours (epirubicin).

All chromatography was carried out at ambient temperature (normally 25°C). Mobile phases were freshly prepared each day and degassed by sonication before use.

2.2.i. Experimental.

The retention behaviour of the studied anthracyclines and their degradation products was investigated, firstly, at a constant water:acetonitrile ratio, between pH 2.0 and 2.8 and secondly at variable acetonitrile concentrations (35 to 60%) at constant pH (pH 2.5). Finally, simultaneous separation of the three parent drugs was investigated.

Vials of doxorubicin, epirubicin (both 10mg), and daunorubicin (20mg) were reconstituted, according to the manufacturers' recommendations, with 5ml, 5ml and 4ml of Water For Injections to yield stock solutions of 2mg/ml, 2mg/ml and 5mg/ml respectively. Standard solutions (200µg/ml) were prepared by dilution of the stock solutions with a Gilson fixed volume pipette.

Partially alkali-degraded solutions were prepared by mixing equal volumes of standard solution and single strength Britton-Robinson buffer pH 11.98 (Appendix I). After thirty minutes an equal amount of 0.1M hydrochloric acid was added to quench the reaction and render the sample more suitable for chromatographic analysis. Partially acid-degraded solutions were prepared by combination of equal amounts of standard solution and single strength Sorenson buffer pH 1.4 (Appendix II). This mixture was placed in a water bath for ten minutes at 80°C then cooled in an ice bath to quench the reaction. After quenching, both acid and alkali-degraded solutions were frozen and stored at -20 °C until assay. For solutions stored at -20°C the difference between inter-assay variability and intra-assay variability was not significant (section 2.3.ii). In addition inter-assay variability was considerably reduced when samples were stored at -20°C and assayed in batches compared to samples which were collected and assayed daily over a period of days.

All solutions were protected from light, with aluminium foil during thawing and prior to assay. Duplicate injections of freshly prepared standard and partially degraded solutions were used to assess the retention behaviour of both parent compounds and their degradation products.

In the study at constant water:acetonitrile ratio the mobile phase consisted of acetonitrile:water (50:50 vol:vol). For the first run the pH of the eluent was adjusted to pH 2.8. On subsequent runs the pH was decreased

in steps of 0.2 units by further addition of 10% orthophosphoric acid.

In the study at constant pH the mobile phase consisted of acetonitrile:water (60:40 vol:vol). For the first run the pH was adjusted to 2.5. The percentage acetonitrile was decreased in steps of 5% and the pH of the mixture was re-adjusted, if necessary, by further addition of 10% orthophosphoric acid.

For the study on the simultaneous separation of the parent anthracyclines injections of freshly prepared standard solutions of each drug (100µg/ml) were used to confirm the expected order of elution. The drug admixture was prepared immediately prior to injection by combination of equal volumes of standard solutions of each drug (300µg/ml) using a Gilson fixed volume pipette.

For the first run the mobile phase consisted of acetonitrile:water (38:62 vol:vol) which was adjusted to pH 2.5. In subsequent runs the percentage acetonitrile was decreased in steps of 2% (38% to 30%) by the addition of calculated volumes of water. The pH of the mobile phase was re-adjusted, if necessary, by further addition of 10% orthophosphoric acid.

The effect of variation of the pH of the eluent (2.5-2.0) at constant water:acetonitrile ratio on the simultaneous separation of the parent anthracyclines was investigated. The mobile phase consisted of acetonitrile:water (30:70 vol:vol). For the first run the pH was adjusted to 2.5. On subsequent runs the pH was decreased in steps of 0.1 units by further addition of 10%

orthophosphoric acid. Separation of the parent compounds was assessed by visual inspection of chromatograms and calculation of HPLC parameters at each concentration of acetonitrile and pH tested.

Ten drops of diethylamine were added to each litre of mobile phase prior to pH adjustment. In all cases the pH of the mobile phase was adjusted with 10% orthophosphoric acid. Elution was isocratic at a flow rate of 1.4ml/min. An equilibration time of twenty minutes, with pumping, was allowed after each change of solvent.

2.2.ii. Results and discussion.

2.2.ii a). The effect of pH of the eluent.

The retention volume of the parent anthracyclines decreased as the pH of the eluent decreased at constant water:acetonitrile ratio (Figure 2.1). Figure 2.2 shows a plot of the logarithm of the column capacity factor ratio (log k') for doxorubicin and its degradation product shown as a function of the acidity of the mobile phase. This plot shows that the log k' of doxorubicin decreased as the pH of the eluent decreased whereas the log k' of the degradation product was unaffected. The pKa of the 3'-amino group in the doxorubicin molecule is about 8.4 (Table 1.3) therefore at pH values between pH 2.0 and 2.8 the majority of the drug would be expected to be ionised. However, the observed decrease in retention volume of the parent compound with a decrease in eluent pH suggested that further ionic effects

control the elution between pH 2.0 and 2.8. As the aglycone degradation products are devoid of the amino sugar moiety the percentage ionised was unaffected by changes in the pH of the mobile phase therefore the retention time remained the same.

Beijnen et al [159] recorded similar results when these compounds were eluted with mobile phases between pH 1.1 and 2.5. In addition these authors observed that an increase in the ionic strength of the eluent at constant pH and water:acetonitrile ratio led to a decrease in the log k' values of the glycosides whereas the retention behaviour of the aglycones was unaffected (Figure 2.3). Those authors concluded that ion-exchange chromatography played an important role in the separation of the glycosides in that system. Ion exchange with the mono-cation, which was formed between pH 1.1 and 2.5, was postulated to involve the free, residual silanol groups of the stationary phase. The differences between the log k' values of the studied glycosides and aglycones, which was the same order of magnitude, was considered to be a measure of the contribution of hydrophobic interactions caused by structural differences, to the chromatographic process.

In this experiment, the presence of diethylamine in the eluent minimised the possibility that ion-exchange was a major feature of the chromatographic process. Separation was more likely to be effected by a partition process which was modified by changes in interaction of the compounds with the column as a result of changes in the pH of the eluent.

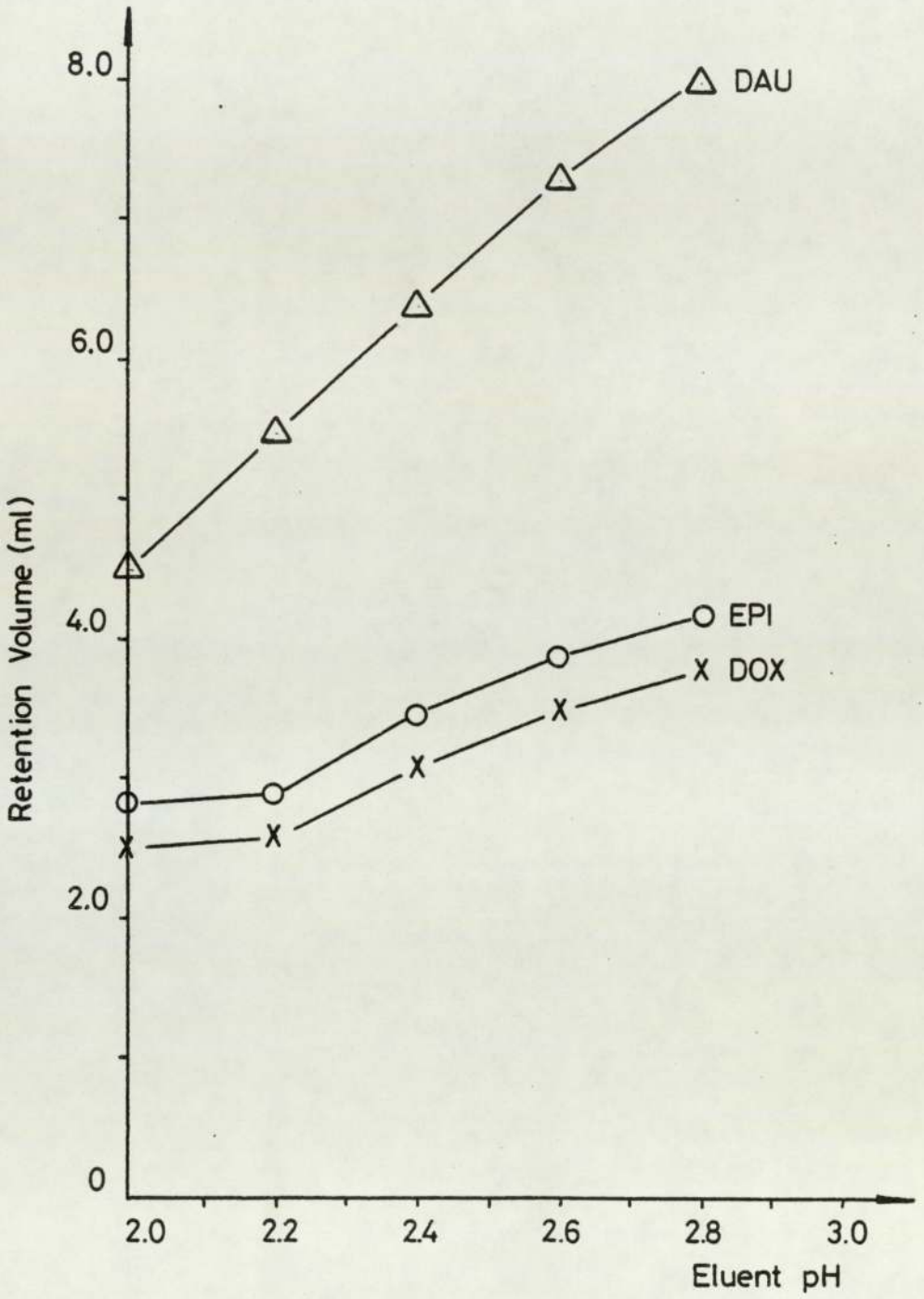


Figure 2.1. The retention volume of doxorubicin, daunorubicin and epirubicin shown as a function of the pH of the eluent (acetonitrile:water 50:50 vol:vol), flow rate: 1.4ml/min., detection: 290nm.

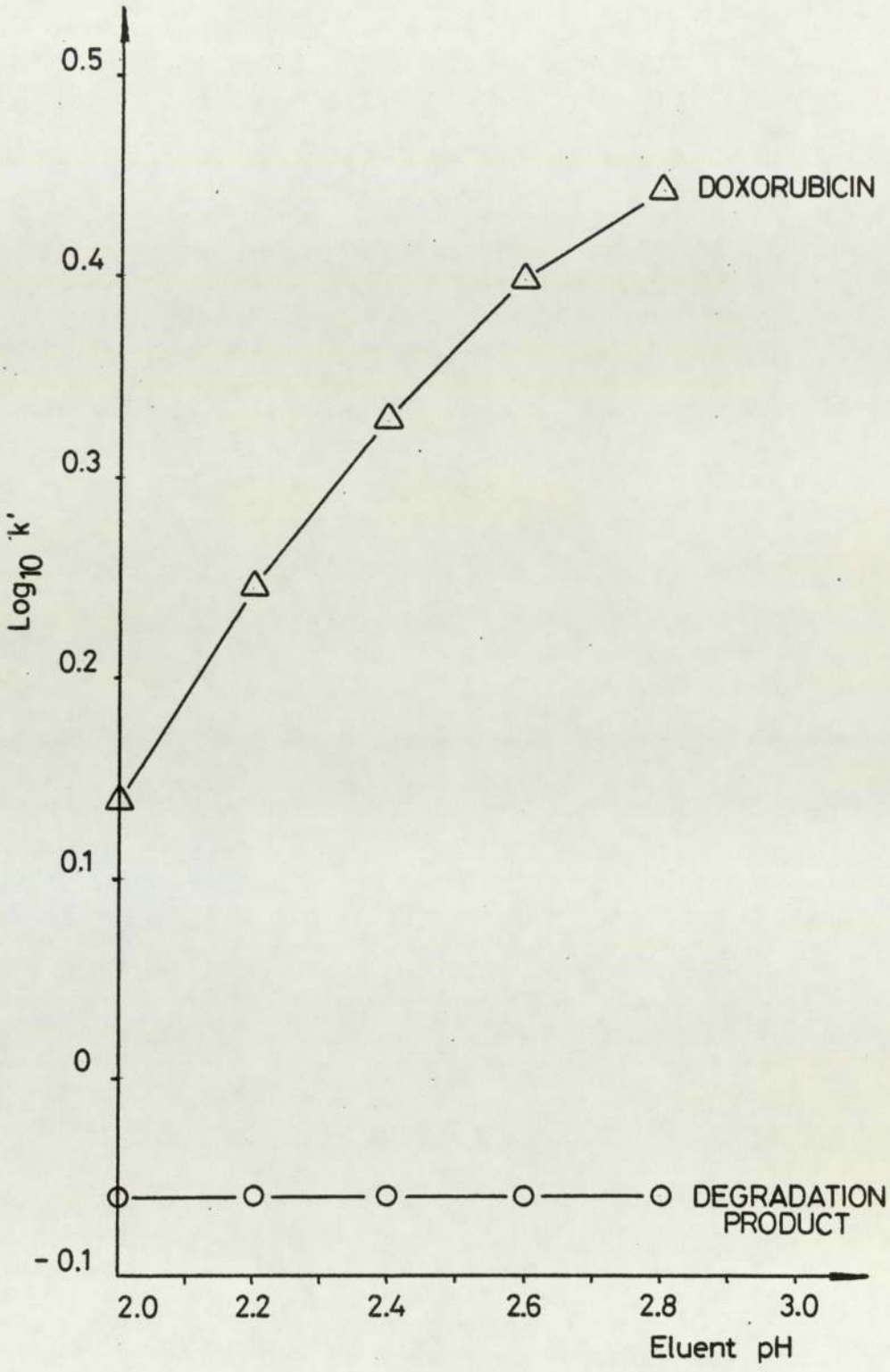


Figure 2.2. The log k' values of doxorubicin and its degradation product (acidic) shown as a function of the pH of the eluent (acetonitrile:water 50:50 vol:vol), flow rate: 1.4ml/min, detection: 290nm.

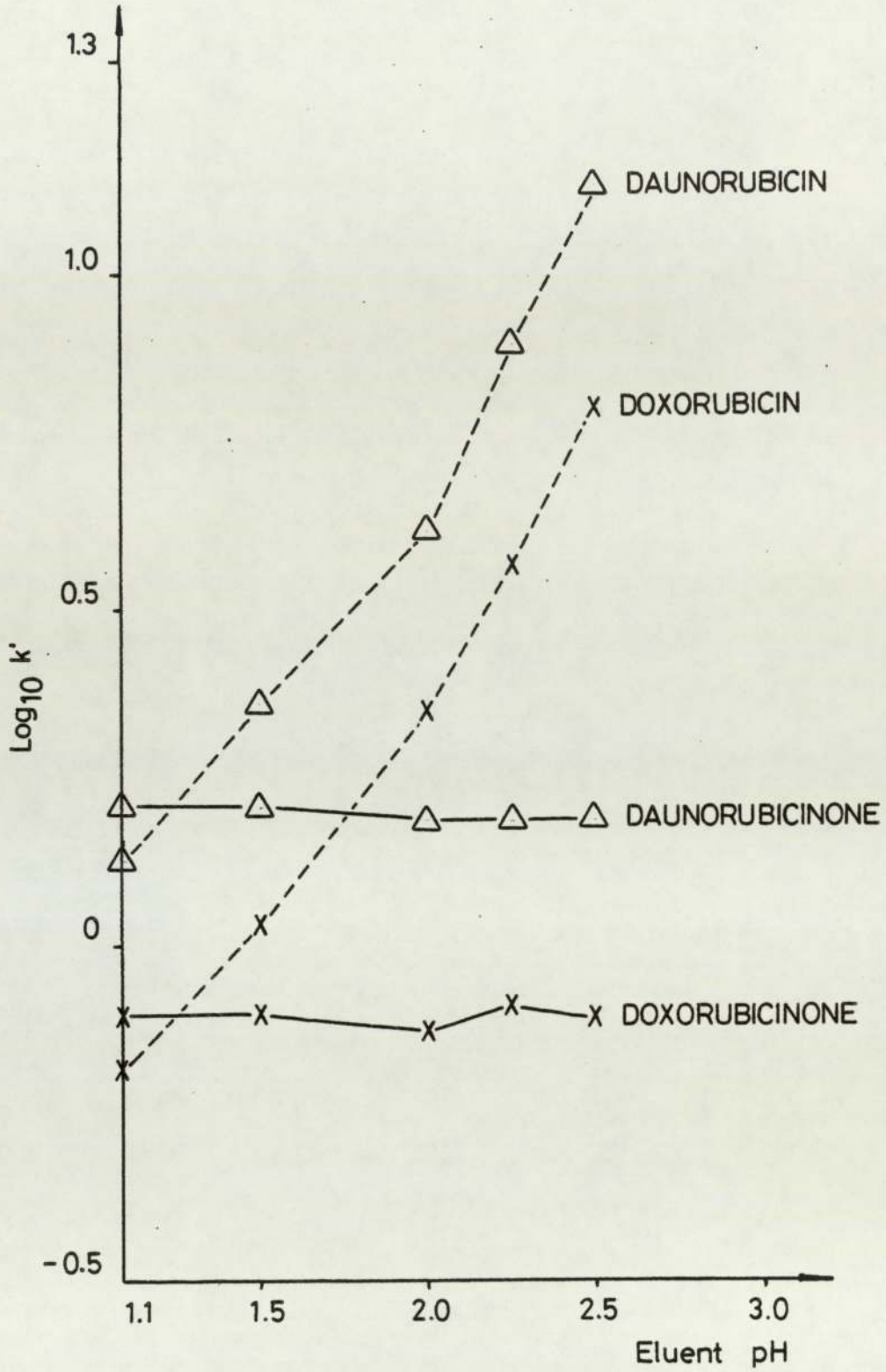


Figure 2.3. The log k' values of doxorubicin, daunorubicin and their aglycones shown as a function of the pH of the eluent (from Beijnen et al [159]). Mobile phase: acetonitrile:water (50:50 vol:vol), column: Lichrosorb 10RP8, flow rate: 1.5ml/min, detection: 254nm.

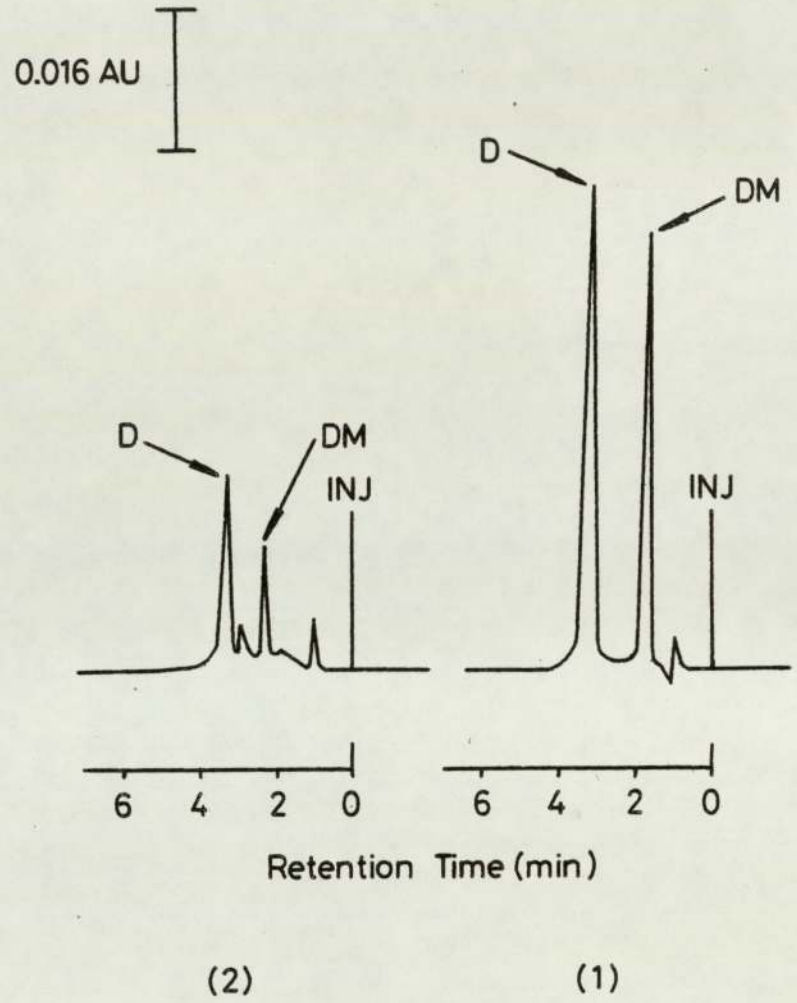


Figure 2.4. Separation of doxorubicin and degradation products from acidic (1) and alkaline (2) solution. Mobile phase: acetonitrile:water (50:50 vol:vol; pH 2.5), flow rate: 1.4ml/min., detection: 290nm, chart speed: 5mm/min., sensitivity: 0.16 AUFS, doxorubicin (D), major degradation product (DM).

Most bonded phase columns only operate between pH 2.0 and pH 7.5 as beyond these values silyl bonds have reduced stability. Columns operated with eluents outside these limits have a shortened life and a tendency to produce poor results, therefore the observations of Beijnen et al [159], at eluent pH values of less than 2 may be of limited use when applied to practical chromatography.

In order to obtain results which were applicable to practical chromatography, eluents between pH 2.0 and 2.8 were used in this study. At an eluent pH of less than 2.5 parent compounds and degradation products were incompletely resolved for both acid and alkali-degraded solutions. At an eluent pH of 2.5 good separation of acid-degraded solutions was obtained but separation of alkali-degraded solutions was incomplete (Figure 2.4).

2.2.ii.b). The effect of the percentage acetonitrile in the eluent.

The retention volumes of doxorubicin, daunorubicin and epirubicin increased as the percentage acetonitrile in the mobile phase decreased at constant pH (Figure 2.5). Figure 2.6. shows the logarithm of the column capacity factor (log k') of doxorubicin and its degradation product (acidic) shown as a function of the percentage acetonitrile in the eluent. This figure shows that the log k' of both the parent compound and the degradation product were almost equally affected by changes in the percentage acetonitrile in the eluent.

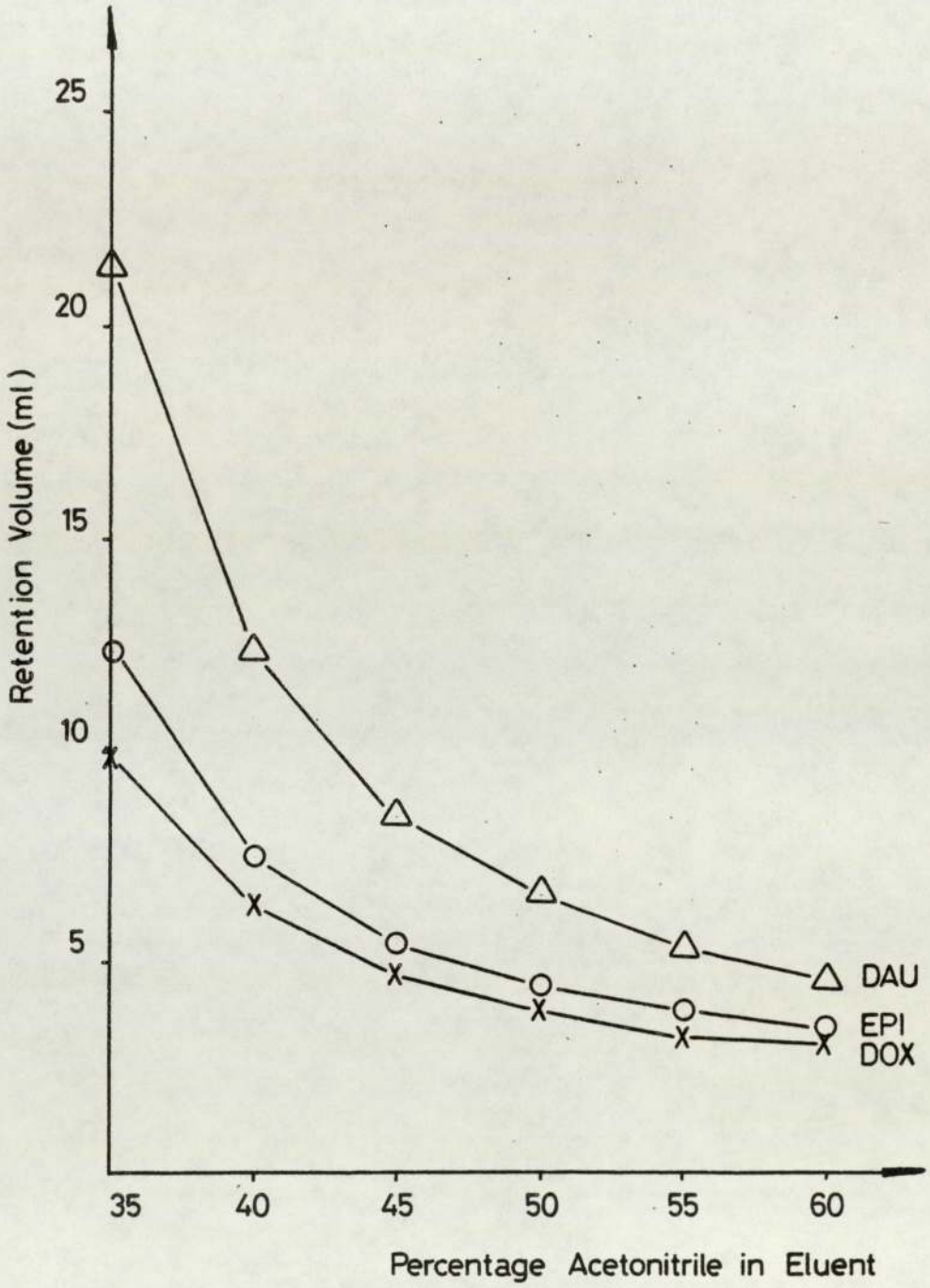


Figure 2.5. The retention volume of doxorubicin, daunorubicin and epirubicin shown as a function of the percentage acetonitrile in the eluent. Mobile phase: acetonitrile:water (variable vol:vol; pH 2.5), flow rate: 1.4 ml/min., detection: 290nm.

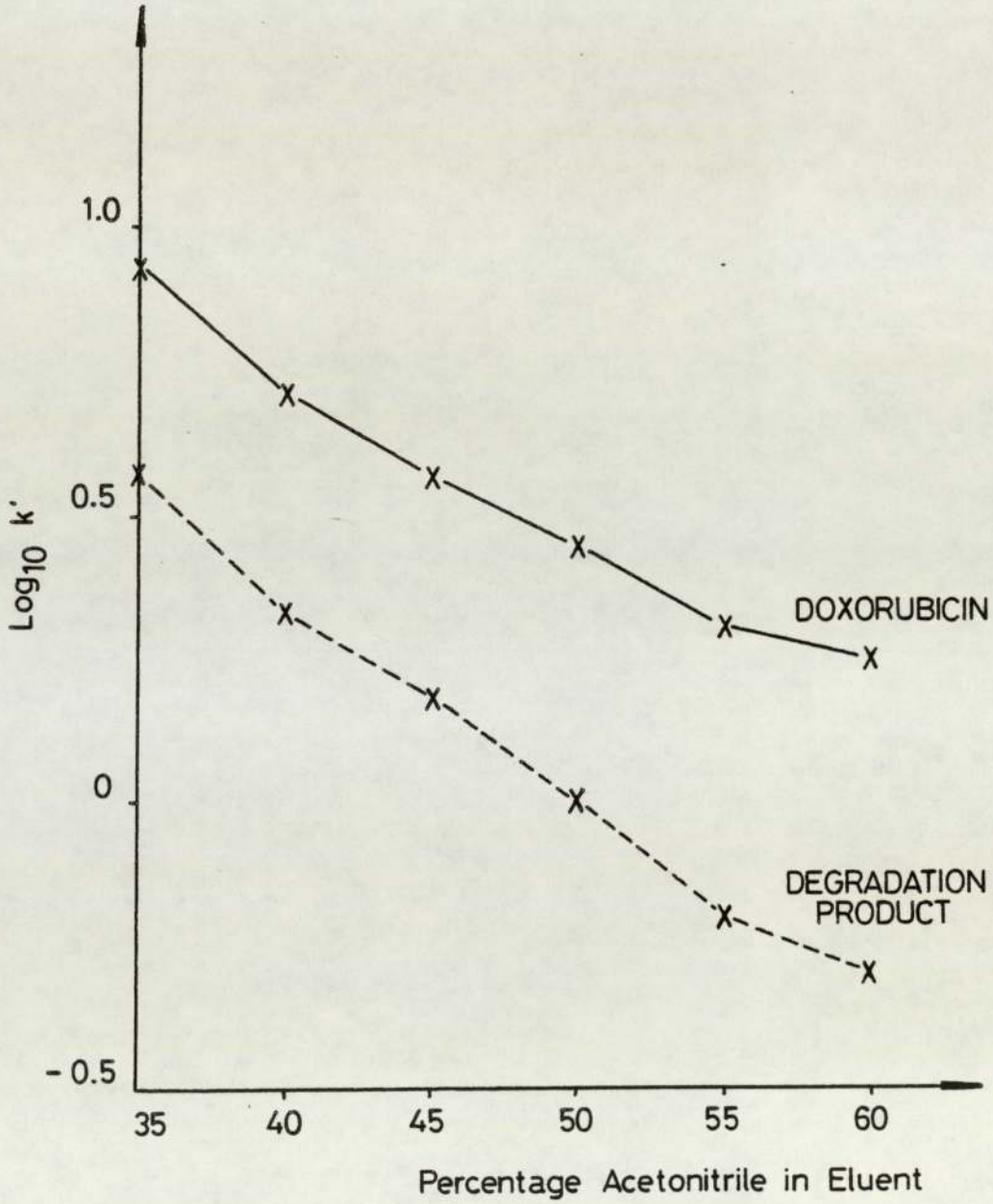


Figure 2.6. The log k' values for doxorubicin and its degradation product (acidic) shown as a function of the percentage acetonitrile in the eluent. Mobile phase: acetonitrile:water (variable vol:vol; pH 2.5), flow rate: 1.4ml/min., detection: 290nm.

Results from the previous two experiments show that systematic adjustment of both the pH and the percentage acetonitrile in the eluent may be used to optimise the resolution of doxorubicin and its degradation products. These principles of separation were also used to optimise the assay conditions for daunorubicin and epirubicin.

For a water:acetonitrile ratio of 50:50 (vol:vol), the optimum eluent pH which allowed separation of acid-degraded solutions, whilst minimising the assay time, was pH 2.5. In order to obtain adequate separation of alkali-degraded solutions a further decrease in percentage acetonitrile was required. The final choice of mobile phase for doxorubicin was acetonitrile:water 40:60 (vol:vol). The separation of doxorubicin and its degradation products under these conditions are shown in Figure 2.7. Epirubicin was assayed with acetonitrile:water 50:50 (vol:vol) and daunorubicin with acetonitrile:water 55:45 (vol:vol). Chromatographic variables for optimum separation of all three compounds are shown in Table 2.1.

Ten drops of diethylamine were added to each litre of mobile phase which was adjusted to pH 2.5 with 10% orthophosphoric acid. For the analysis of doxorubicin the flow rate was maintained at 1.4ml/min, for epirubicin 1.3ml/min and for daunorubicin 1.5ml/min. In all cases detection was performed at 290nm.

drug admixture	Rv1	Rv2	k'1	k'2	α	Rs
dox (pH 1.4)	6.4	3.3	5.40	2.30	2.35	1.38
dox (pH 11.98)	6.4	2.9	5.40	1.90	2.84	2.33
epi (pH 1.4)	4.3	2.1	3.30	1.10	3.00	1.26
epi (pH 11.98)	4.2	2.3	3.20	1.30	2.46	2.53
dau (pH 1.4)	5.4	2.9	5.75	2.60	2.19	1.25
dau (pH 11.98)	5.3	1.8	5.63	1.25	4.50	2.33

Table 2.1. Chromatographic variables calculated for doxorubicin, daunorubicin and epirubicin and major degradation products from acidic and alkaline solution. Mobile phase acetonitrile: water (doxorubicin 40:60, epirubicin 50:50, daunorubicin 55:45; all pH 2.5), flow rate: 1.4ml/min., 1.3ml/min., and 1.5ml/min. respectively, detection: 290nm.

Key:

Rv1 = retention volume of parent compound
Rv2 = retention volume of major degradation product
k'1 = capacity factor for parent compound
k'2 = capacity factor for major degradation product
α = separation factor
Rs = resolution of parent compound and major degradation product

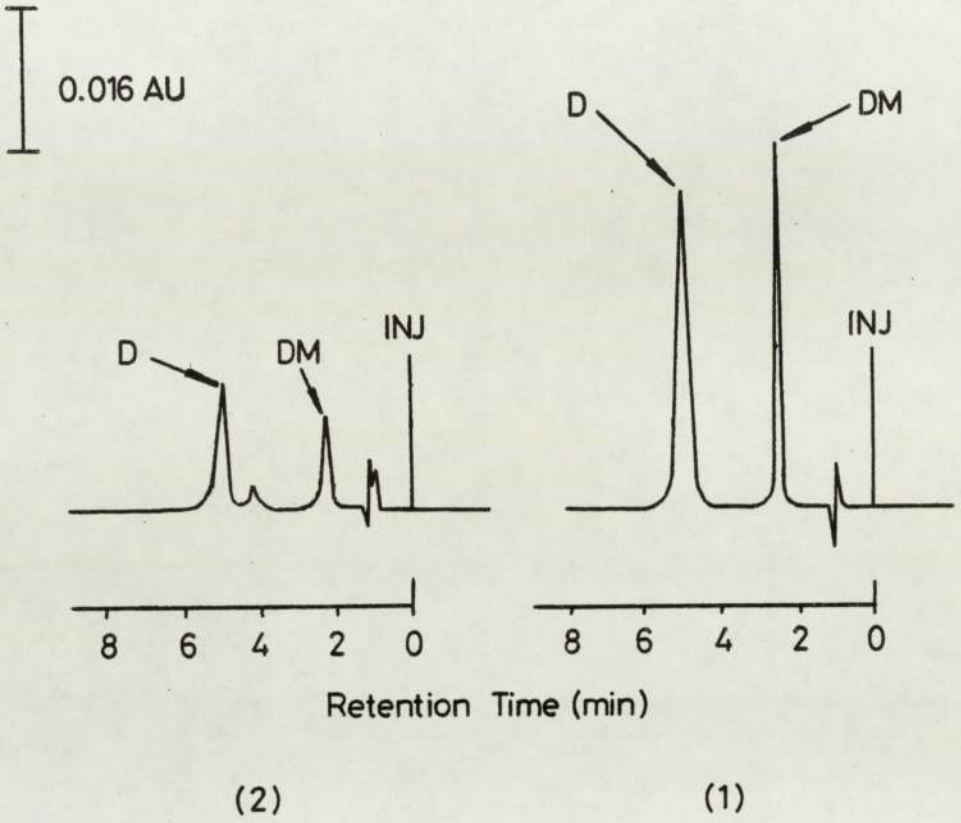


Figure 2.7. Separation of doxorubicin and degradation products from acidic (1) and alkaline (2) solution. Mobile phase acetonitrile:water (40:60 vol:vol; pH 2.5), flow rate: 1.4ml/min., detection: 290nm, chart speed 5mm/min., sensitivity: 0.16 AUFS, doxorubicin (D), major degradation product (DM).

2.2.ii.c). Simultaneous separation of the parent compounds.

The daunorubicin peak, which was the last to elute from the column, was well separated from the doxorubicin and epirubicin peaks at an acetonitrile concentration of 38% (pH 2.5) but the doxorubicin and epirubicin peaks were only partially resolved. As the percentage acetonitrile in the eluent was decreased the retention volume of all three

compounds increased (Figure 2.8) until at 30% acetonitrile the doxorubicin and epirubicin peaks were well resolved but the daunorubicin peak was very broad and unquantifiable.

As the pH of the mobile phase was decreased (2.5 to 2.0), at a constant water:acetonitrile ratio of 30:70 (vol:vol), the retention volume of all three compounds decreased (Figure 2.9). Figure 2.9 also shows that daunorubicin is more sensitive to changes in eluent pH than either doxorubicin or epirubicin. A dramatic improvement in the shape of the daunorubicin peak was observed as the retention volume decreased which was reflected by an increase in the number of theoretical plates (N) for this peak, from 2600 at pH 2.5 to 4000 at pH 2.0. The retention volumes of doxorubicin and epirubicin were equally affected therefore no loss of resolution of these peaks was observed. However, the efficiency of the system increased as the pH of the eluent decreased. The number of theoretical plates increased from 3500, at pH 2.5, to 5000 at pH 2.0 for the doxorubicin peak and from 3100 to 4550 for the epirubicin peak.

These data indicate that doxorubicin, daunorubicin and epirubicin may be simultaneously separated and quantified with reasonable efficiency using a mobile phase which consisted of acetonitrile:water 30:70 (vol:vol) with ten drops of diethylamine per litre adjusted to pH 2.0 with 10% orthophosphoric acid (vol:vol) (Figure 2.10). The important chromatographic variables for this separation are shown in Table 2.2.

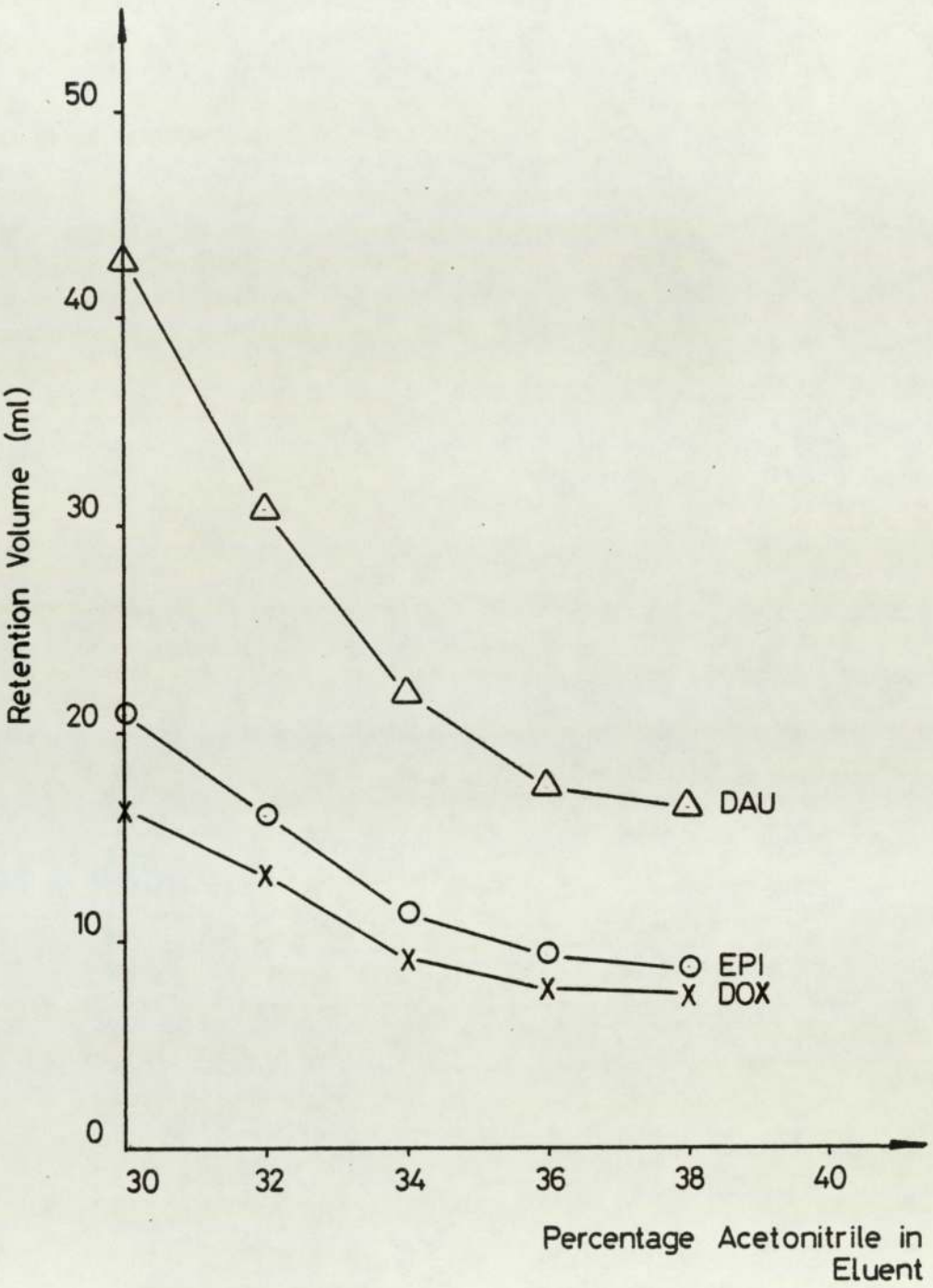


Figure 2.8. The retention volume of doxorubicin, daunorubicin and epirubicin shown as a function of the percentage acetonitrile in the eluent. Mobile phase: acetonitrile: water; pH 2.5, flow rate: 1.4ml/min., detection: 290nm.

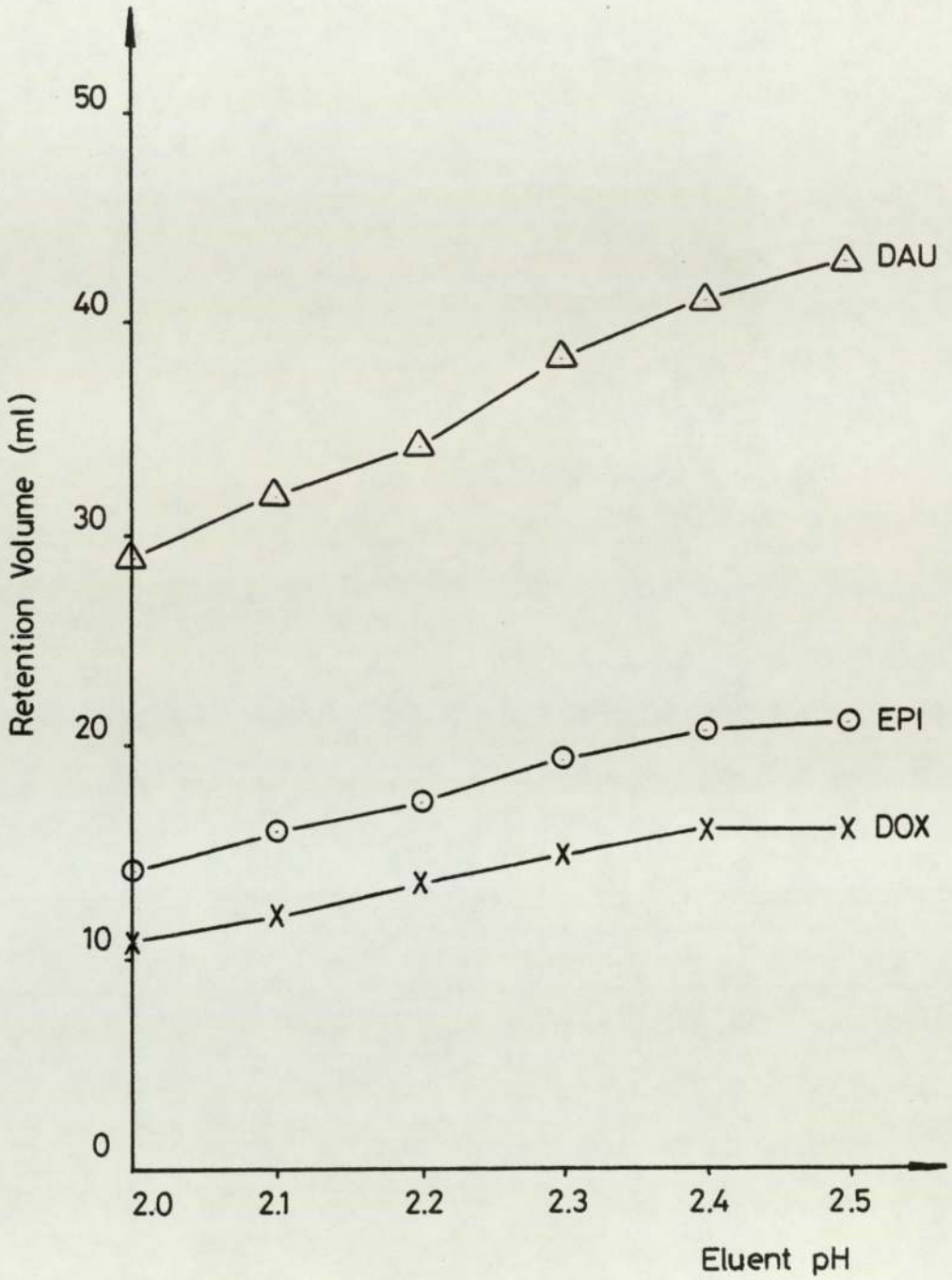


Figure 2.9. The retention volume of doxorubicin, daunorubicin and epirubicin shown as a function of the pH of the eluent. Mobile phase: acetonitrile:water (30:70 vol:vol), flow rate: 1.4ml/min., detection: 290nm.

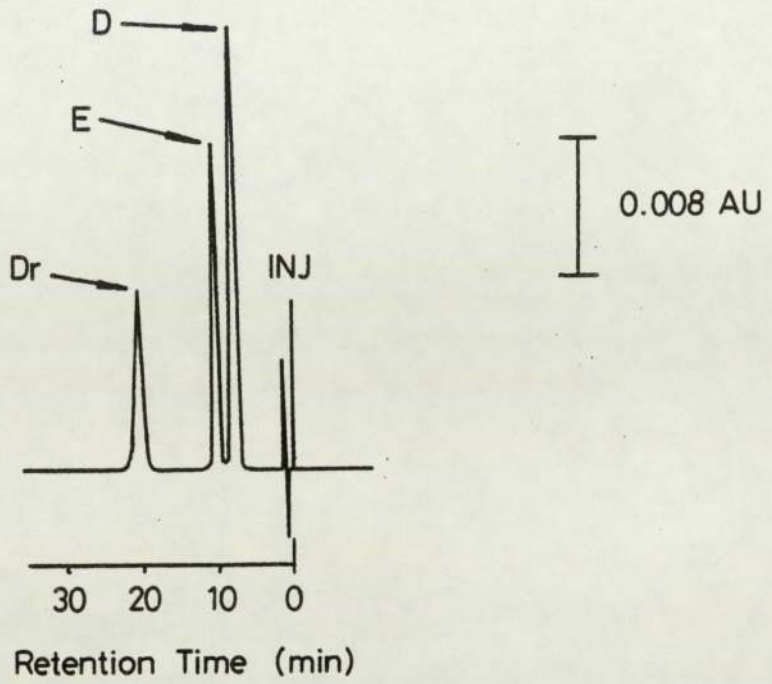


Figure 2.10. Chromatographic separation of doxorubicin (D), epirubicin (E) and daunorubicin (Dr). Mobile phase: acetonitrile:water (30:70 vol:vol; pH 2.0), flow rate: 1.4ml/min., detection: 290nm, chart speed: 1mm/min., sensitivity: 0.08 AUFS.

DRUG	Rv1	k1'	DOX		EPI		DAU	
			α	Rs	α	Rs	α	Rs
DOX	10.9	6.52	-	-	1.35	2.20	2.90	10.30
EPI	14.2	8.79	1.35	2.20	-	-	2.16	8.46
DAU	29.0	19.0	2.90	10.30	2.16	8.46	-	-

Table 2.2. Chromatographic variables calculated from the adjusted retention volumes from Figure 2.10.

Key:

Rv1 = retention volume
k1' = capacity factor
α = separation factor
Rs = resolution

2.3. Application of the developed system.

2.3.i. Experimental.

Vials of each drug were reconstituted, according to the manufacturers' recommendations, and standard solutions (50μg/ml to 500μg/ml) were produced after dilution with a Gilson fixed volume pipette (section 2.2.i.) . Samples of these solutions were then injected onto the chromatograph. Plots of peak height versus concentration were constructed daily prior to assay of experimental samples. Each data point was the mean value obtained from six replicate injections.

Inter- and intra-day variability of HPLC assays were evaluated for doxorubicin. A bulk standard solution (100μg/ml) (section 2.2.i.) was freshly prepared and divided between twelve identical glass containers. These containers were sealed with plastic screw caps, frozen and stored at -20°C until assay.

On six consecutive days, two of these containers were thawed at ambient temperature and six replicates injected onto the chromatograph. The concentration of the bulk solution was calculated by interpolation of data on calibration curves which were constructed daily.

2.3.ii. Results and discussion.

Peak height was proportional to doxorubicin

concentration between 10µg/ml and 50µg/ml but calibration curves constructed between 10µg/ml and 500µg/ml were not linear as peak broadening occurred at concentrations greater than 50µg/ml. Drug concentration was proportional to peak area for all concentrations ranges tested therefore a peak area estimate (peak height multiplied by peak width at one tenth height) was used for quantitation in all experimental work. Standard curves based on these calculated peak areas were linear for all three drugs in the range 0 to 500µg/ml as shown in Figures 2.11, 2.12, and 2.13.

Results from an analysis of variance for intra- and inter-assay variability are shown in Table 2.3. The mean concentration calculated from the values in this Table was 95.3 µg/ml, the standard deviation (SD) was 2.5 and the coefficient of variation was 2.6% therefore 95% of the observations lie within 2SD of the mean that is 95.3 ± 5.0 µg/ml.

From tables [180] the critical F value was 2.53 at the 5 percent level of significance. Since the calculated F value from the analysis of variance (Table 2.3) was much less than 2.53 it may be concluded that the variance between samples was not significantly greater than the variance within samples. This suggests that freezing of experimental samples and storage at -20° C did not influence the reproducibility of the assay.

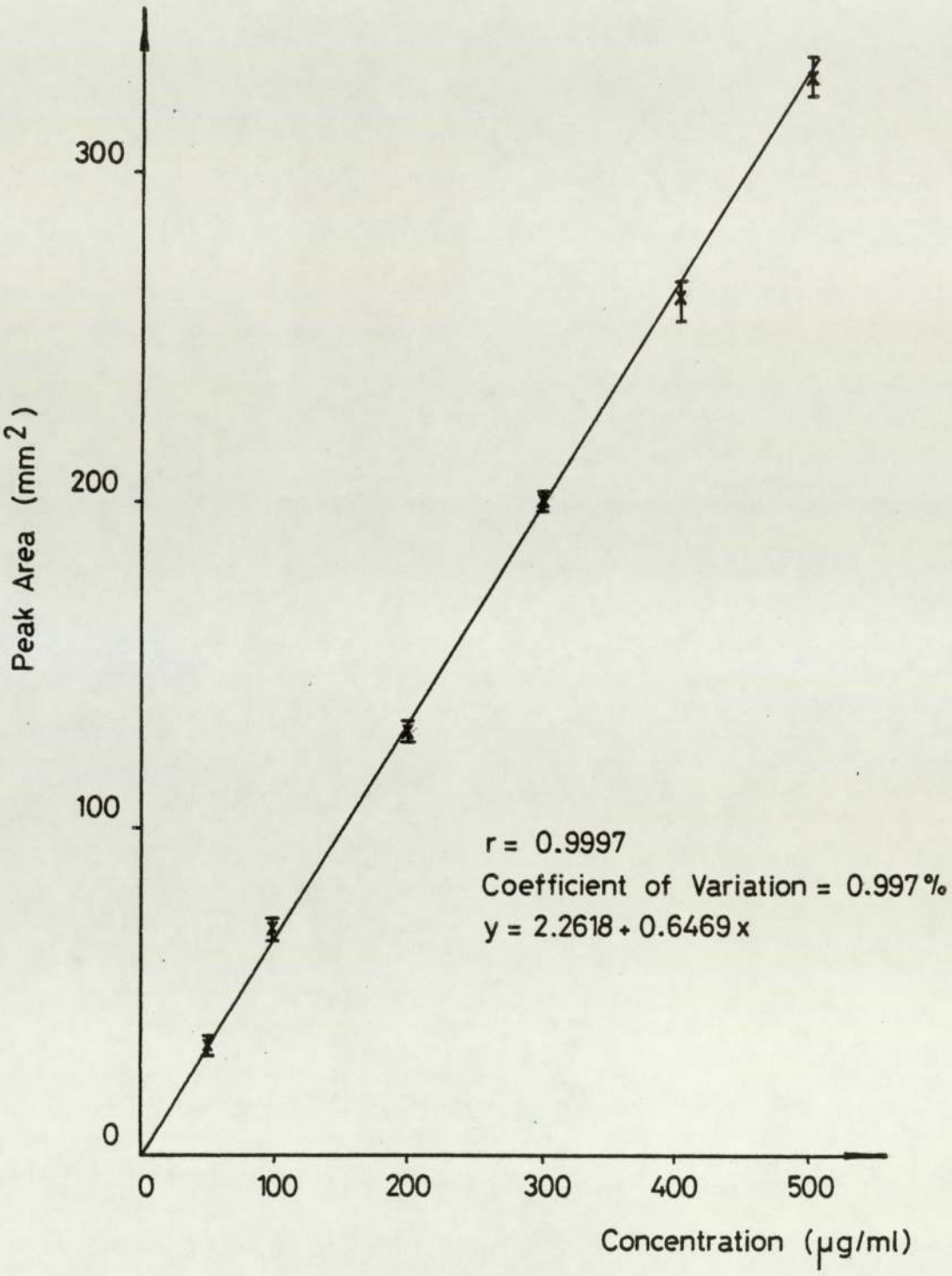


Figure 2.11. Calibration curve for doxorubicin.

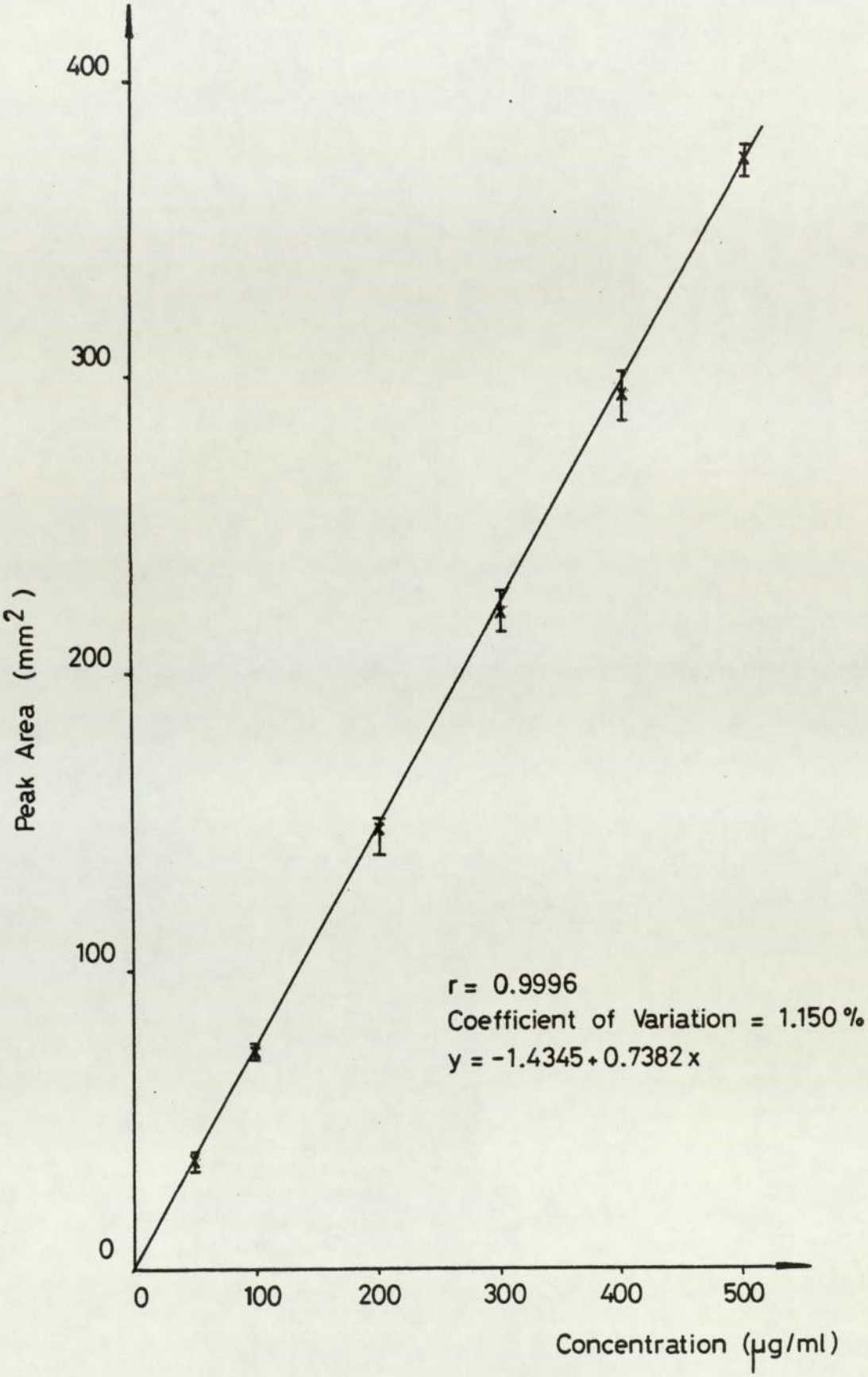


Figure 2.12. Calibration curve for epirubicin.

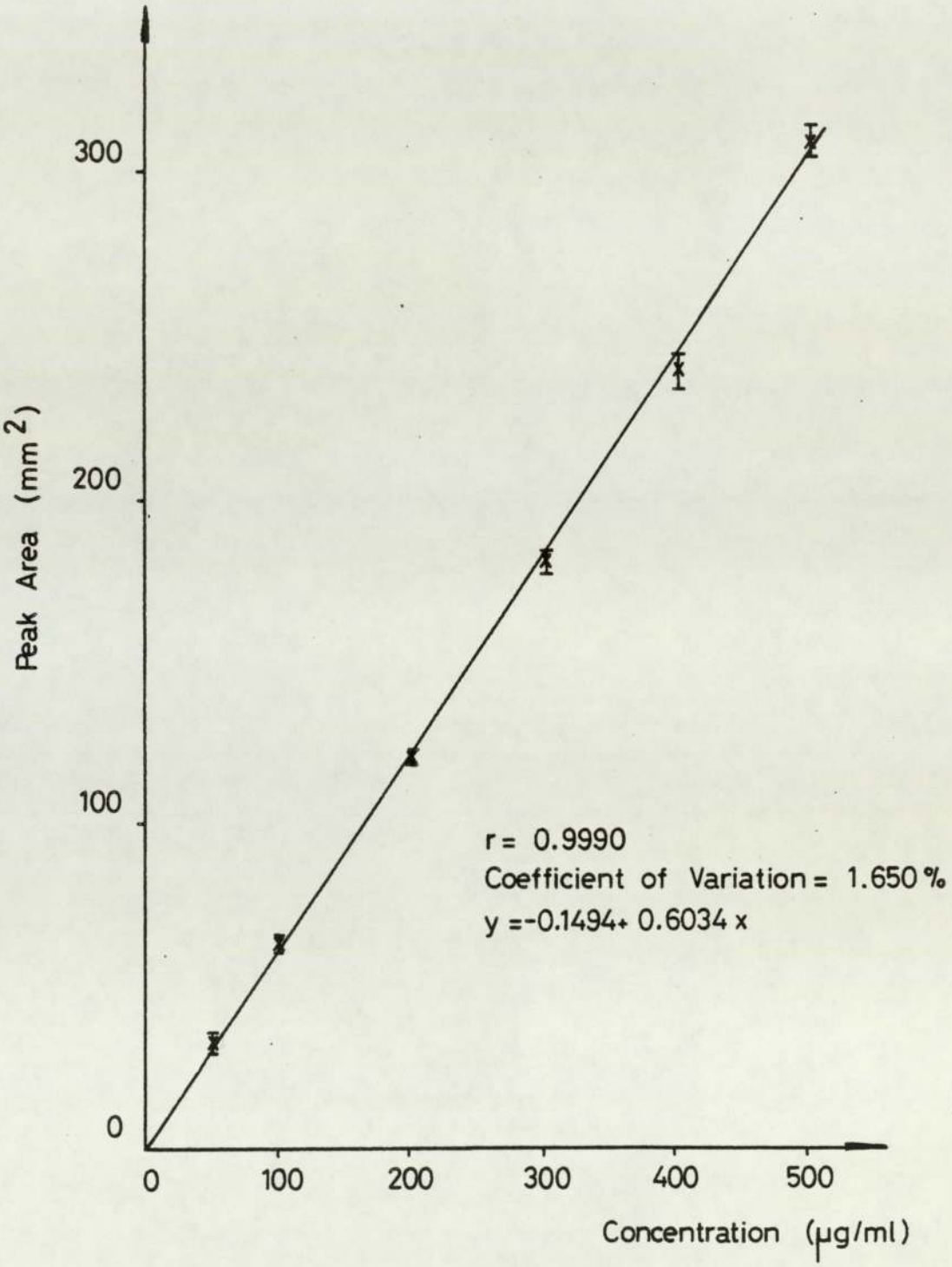


Figure 2.13. Calibration curve for daunorubicin.

		REPLICATES (µg/ml)					
DAY OF ASSAY	1	97.9	98.4	98.4	94.5	94.1	92.6
	2	98.4	99.9	97.8	99.9	94.2	93.7
	3	93.4	96.6	95.7	93.4	97.6	97.6
	4	89.3	95.4	96.4	96.9	91.7	92.6
	5	93.9	93.5	95.8	97.3	95.4	92.0
	6	95.3	96.1	96.1	90.6	93.2	96.1

Variability

SOURCE	SUM SQUARES	DEGREES OF FREEDOM
Columns	39.5625	5
Residuals	186.7813	30
Total	226.3438	35

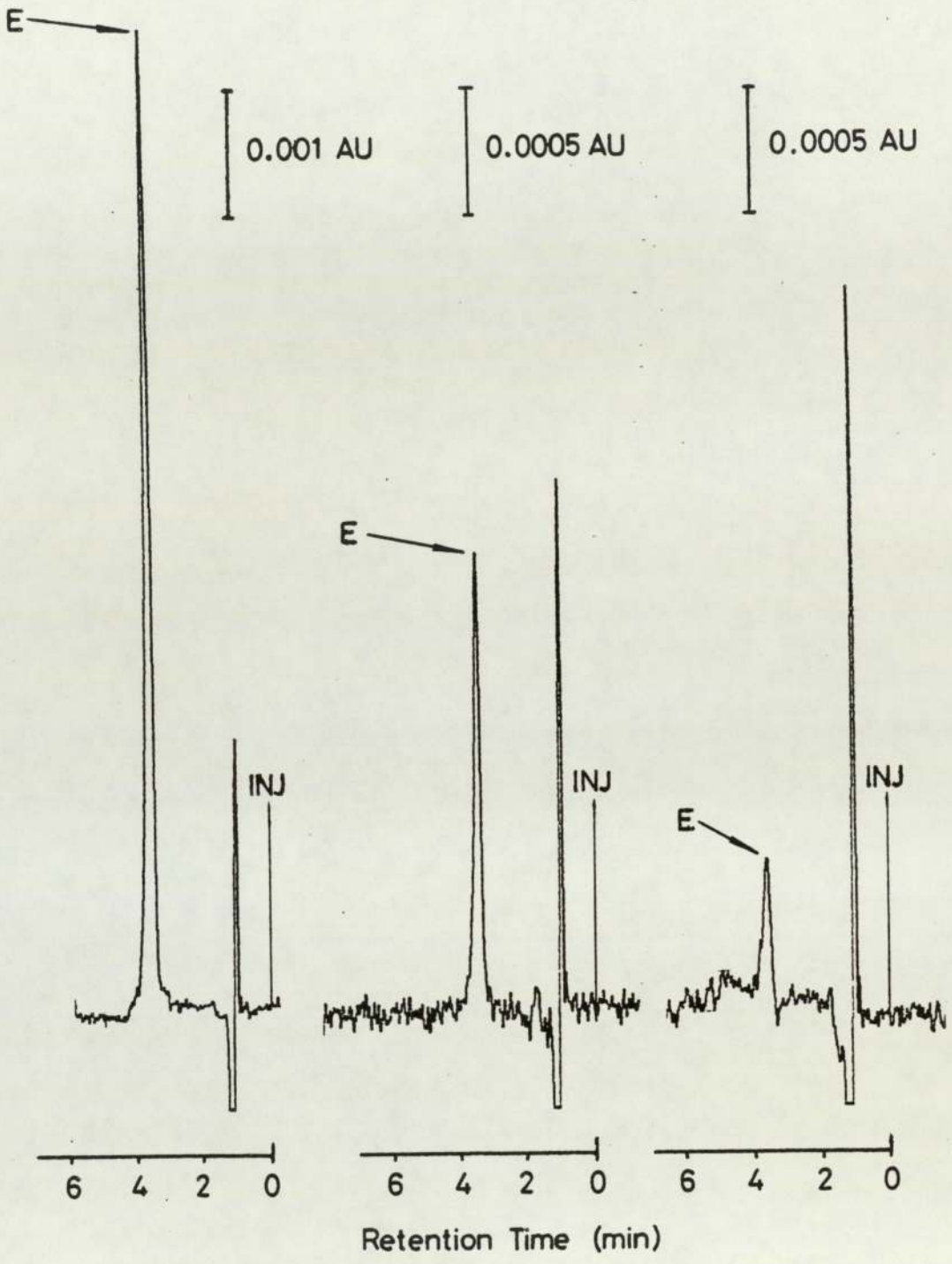
SOURCE	MEAN SQUARE	F-RATIO (DF1,DF2)
Columns	7.9125	1.270872 (5,30)
Residuals	6.2260	

Table 2.3. Analysis of variance data (one-way with replicates) for doxorubicin used to assess the intra- and inter-assay variability of the HPLC assay.

Traditionally, for most compounds including the anthracyclines, ultraviolet (UV) detectors were operated at 254nm during HPLC analysis, as early detectors used mercury lamps which radiated at this wavelength. Poochikian et al [173] observed that the peak heights of the decomposition products of doxorubicin were relatively small compared to the initial height of the doxorubicin peak. These authors postulated that decomposition products either had a weaker chromophore or that their wavelength of maximum absorption (λ max) was shifted to other wavelengths. In order to investigate this hypothesis the ultraviolet spectra of doxorubicin in 0.1M hydrochloric acid and 0.1M sodium hydroxide were obtained.

DOX ADMIXTURE	CONC. (µg/ml	REFERENCE SAMPLE	λ max VALUES
DISTILLED WATER	10	DISTILLED WATER	234, 252, 290, 480, 495, 532nm.
0.1M HYDROCHLORIC ACID	10	0.1M HYDROCHLORIC ACID	232, 252, 290, 480, 495, 532nm
0.1M SODIUM HYDROXIDE	10	0.1M SODIUM HYDROXIDE	234, 252, 290, 545, 585nm.

Table 2.4. Details of ultraviolet spectra obtained for doxorubicin.



a) 5µg/ml 0.01 AUFS

b) 1µg/ml 0.005 AUFS

c) 100ng/ml 0.005 AUFS

Figure 2.14. High performance liquid chromatogram obtained to calculate the sensitivity of the HPLC assay. Mobile phase: acetonitrile:water (50:50 vol:vol; pH 2.5), flow rate: 1.3ml/min., detection: 290nm, chart speed: 5mm/min., epirubicin (E).

The spectra in 0.1M hydrochloric acid and distilled water were similar but the spectrum obtained in 0.1M sodium hydroxide was significantly different (Table 2.4). Firstly, the peak which was present at 480nm in the spectra obtained in distilled water and 0.1M hydrochloric acid was not apparent in the spectrum obtained in 0.1M sodium hydroxide. Secondly, peaks at 495nm and 532nm in the spectrum obtained in distilled water were shifted to 545 and 585nm respectively. Data from these spectra indicated that 290nm was a suitable wavelength for assay of both parent anthracyclines and degradation products.

For an operational wavelength of 290nm and an injection volume of 10µl, the limit of detection for doxorubicin, daunorubicin and epirubicin, defined as three times the base line noise, was 100ng/ml (Figure 2.14). The minimum quantifiable concentration, defined as ten times the level of baseline noise, was 350ng/ml.

The assays developed for these anthracyclines were used to quantitate the rate of degradation in light, at extremes of temperature and pH and finally in infusion fluids. The results from these stability studies are presented in the following chapters.

Chapter Three

Photodegradation of doxorubicin, daunorubicin and epirubicin.

3.1. Introduction.

The aim of the following studies was to investigate and compare the degradation kinetics of doxorubicin, daunorubicin and epirubicin in fluorescent light. The conditions which facilitated or prevented photodegradation were identified by investigation of the effect of drug concentration, the solvent pH and the type of storage container on the rate of photodegradation.

3.2. Experimental.

Initially, the photodegradation of doxorubicin in aqueous solution was investigated at 500, 250, 100, 50, 25 and 10µg/ml in clear glass, amber glass and plastic (polyethylene) bottles. Photodegradation was compared to degradation in control solutions stored in the dark. The photodegradation of daunorubicin and epirubicin was also investigated in aqueous solution in clear glass only between 10µg/ml and 100µg/ml. On completion of these studies the effect of increased solvent pH on the rate of photodegradation of doxorubicin was investigated.

Vials of doxorubicin, epirubicin and daunorubicin were reconstituted, with Water For Injections (section 2.2.i.) to yield stock solutions of 2mg/ml, 2mg/ml and 5mg/ml respectively. Standard solutions (10µg/ml to 500µg/ml) were prepared by dilution of the stock solutions. Photodegradation of these standard solutions was

investigated directly after transfer to the experimental containers. For the study at increased pH, admixtures were prepared by combination of equal volumes of a standard solution of doxorubicin (50μg/ml) with single strength Tris buffer (pH 7.2 and 8.0) (Appendix III).

Duplicates of each container were sealed with plastic screw caps and stored at ambient temperature (normally 25°C), either covered with aluminium foil in a darkened room (doxorubicin only) or exposed to room light. In the room light studies constant illumination was provided by four 65/80 watt ceiling fluorescent tubes (Thorn EMI) mounted approximately one metre above the samples which were placed upright on the laboratory bench.

For the study in aqueous solution, pH 6.1, samples (2ml aliquots) were taken initially and at twenty four hour intervals over a period of 168 hours. For the study in Tris buffer samples were taken initially then at two hourly intervals over a period of eight hours from the containers stored in room light and at twenty four hour intervals for 168 hours from containers stored in the dark. An equal volume of 0.1M hydrochloric acid was added to each Tris buffer sample to quench the reaction and render the sample more suitable for chromatographic analysis.

Samples were immediately frozen, stored at -20°C and thawed at ambient temperature immediately before assay. All samples were protected from light prior to initiation of the study, during thawing and prior to assay. Duplicates of each sample (10μl) were injected onto the column and eluted with

the mobile phases defined in section 2.2.ii.b.

Chromatograms were inspected for evidence of degradation products. Concentrations remaining were determined by interpolation of peak areas (section 2.3.ii) on calibration curves which were constructed daily. All concentrations, which were expressed as percentages of the initial value obtained at time 0, represented the mean of duplicate values. Significant degradation was defined as a loss of equal to or greater than 10% of the original concentration. Where possible the time for 10% degradation to occur, the t90% value, was calculated. Data were evaluated for first order kinetics by construction of plots of the natural logarithm of the percentage parent drug remaining versus time using the equation;

$$\ln x_t = \ln x_o - k_{obs} . t$$

where x_t = concentration remaining at time t

x_o = initial concentration

k_{obs} = the observed rate constant

The rate constant, k_{obs} , was calculated by linear regression analysis of the first order plot. Differences in the extent of degradation of the analysed samples of doxorubicin, daunorubicin and epirubicin were tested for statistical significance by means of a two-way analysis of variance with replicates. Where it was necessary only to test the difference between two means for significance Student's t Test was employed. The pooled standard deviation

for the two test samples and the standard error of the difference was calculated according to the usual equations [181].

3.3. Results and discussion.

Plots of the natural logarithm of the percentage parent drug remaining versus time were linear ($r => 0.999$) which indicated that photodegradation followed first order kinetics. The first order plot for epirubicin (Figure 3.1) showed that rapid photolysis occurred at concentrations below 100µg/ml. Plots of the rate constant, k_{obs}, for photodegradation versus the reciprocal of the initial drug concentration were linear indicating that the rate of photodegradation was inversely proportional to the initial drug concentration (Figure 3.2).

Table 3.1 shows the percentage doxorubicin remaining after exposure to room light for 168 hours and Tables 3.2.a. and 3.2.b. show the rate constants for photodegradation of these solutions. These data show that rapid photodegradation occurred in clear glass containers. The amber glass and opaque polyethylene containers afforded some protection from light but significant drug loss (greater than or equal to 10%) was observed at 25µg/ml and 10µg/ml after approximately 72 hours and 24 hours respectively. At concentrations equal to or greater than 100µg/ml little or no photodegradation occurred in either glass or plastic containers.

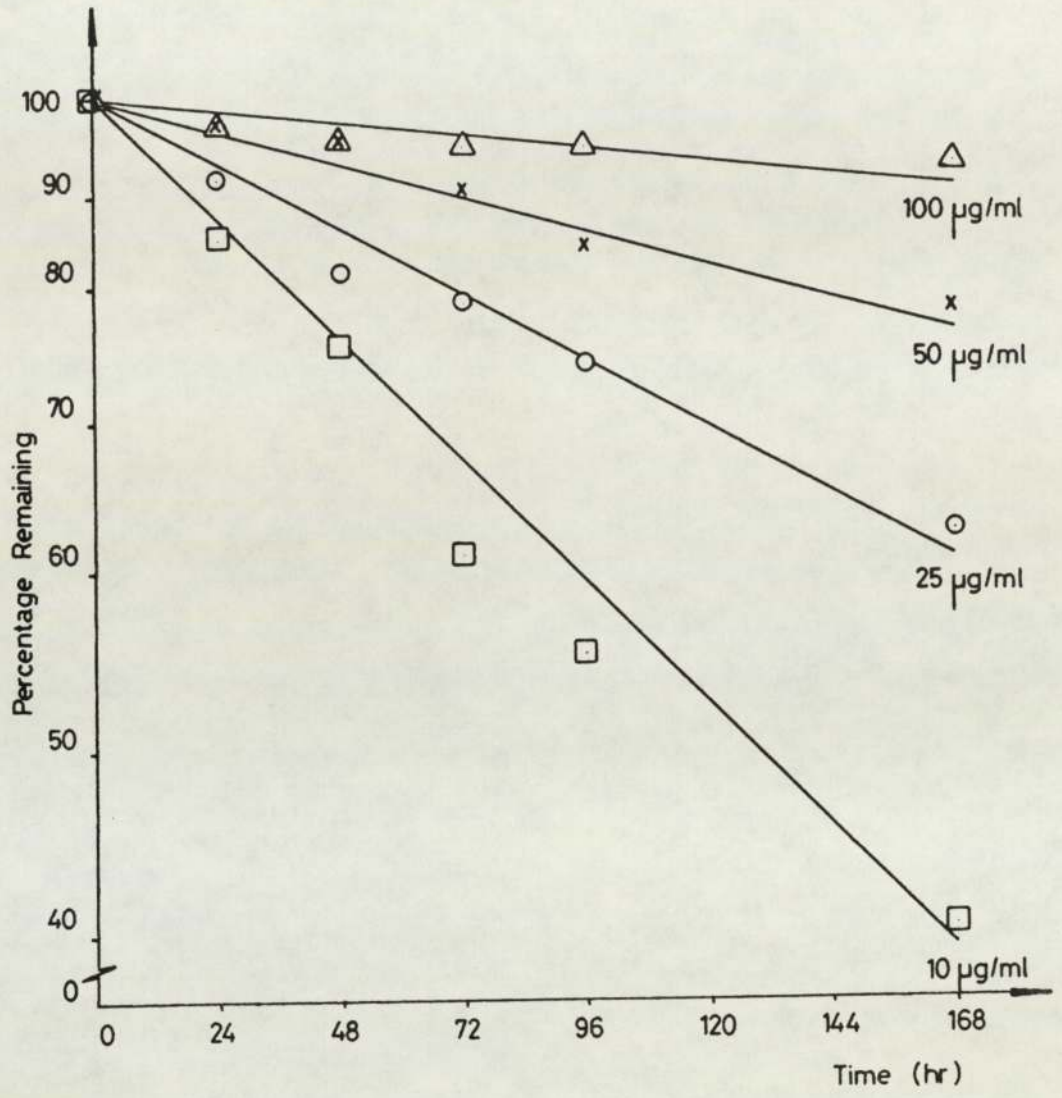


Figure 3.1. Natural logarithm of the percentage epirubicin remaining versus time for photodegradation in clear glass.

CONTAINER	PERCENTAGE DOXORUBICIN REMAINING ($\bar{x}$ ± SD n = 3)					
	DRUG CONCENTRATION µg/ml					
	500	250	100	50	25	10
CLEAR GLASS	98.6 ± 0.5	94.8 ± 0.6	93.3 ± 1.4	84.9 ± 0.6	61.5 ± 1.5	31.8 ± 1.4
AMBER GLASS	96.1 ± 0.7	97.3 ± 0.5	96.4 ± 1.1	94.6 ± 1.6	82.1 ± 1.6	35.8 ± 1.6
POLYETHYLENE	98.8 ± 0.8	101.8 ± 0.8	96.8 ± 0.7	96.9 ± 1.7	83.3 ± 1.5	39.5 ± 1.8

Table 3.1. Percentage doxorubicin remaining after exposure of an aqueous solution to room light for 168 hours.

CONTAINER	RATE CONSTANT k (hr^{-1})		
	500µg/ml	250µg/ml	100µg/ml
CLEAR GLASS	1.29×10^{-4}	3.28×10^{-4}	3.93×10^{-4}
AMBER GLASS	2.49×10^{-4}	1.01×10^{-4}	2.42×10^{-4}
POLYETHYLENE	7.93×10^{-5}	1.12×10^{-4}	2.03×10^{-4}

Table 3.2.a. Rate constants for photodegradation of doxorubicin in aqueous solution between 500µg/ml and 100µg/ml.

CONTAINER	RATE CONSTANT k (hr^{-1})		
	50µg/ml	25µg/ml	10µg/ml
CLEAR GLASS	1.01×10^{-3}	2.85×10^{-3}	6.50×10^{-3}
AMBER GLASS	2.86×10^{-4}	1.14×10^{-3}	5.99×10^{-3}
POLYETHYLENE	1.47×10^{-4}	1.02×10^{-3}	5.05×10^{-3}

Table 3.2.b. Rate constants for photodegradation of doxorubicin in aqueous solution between 50µg/ml and 10µg/ml.

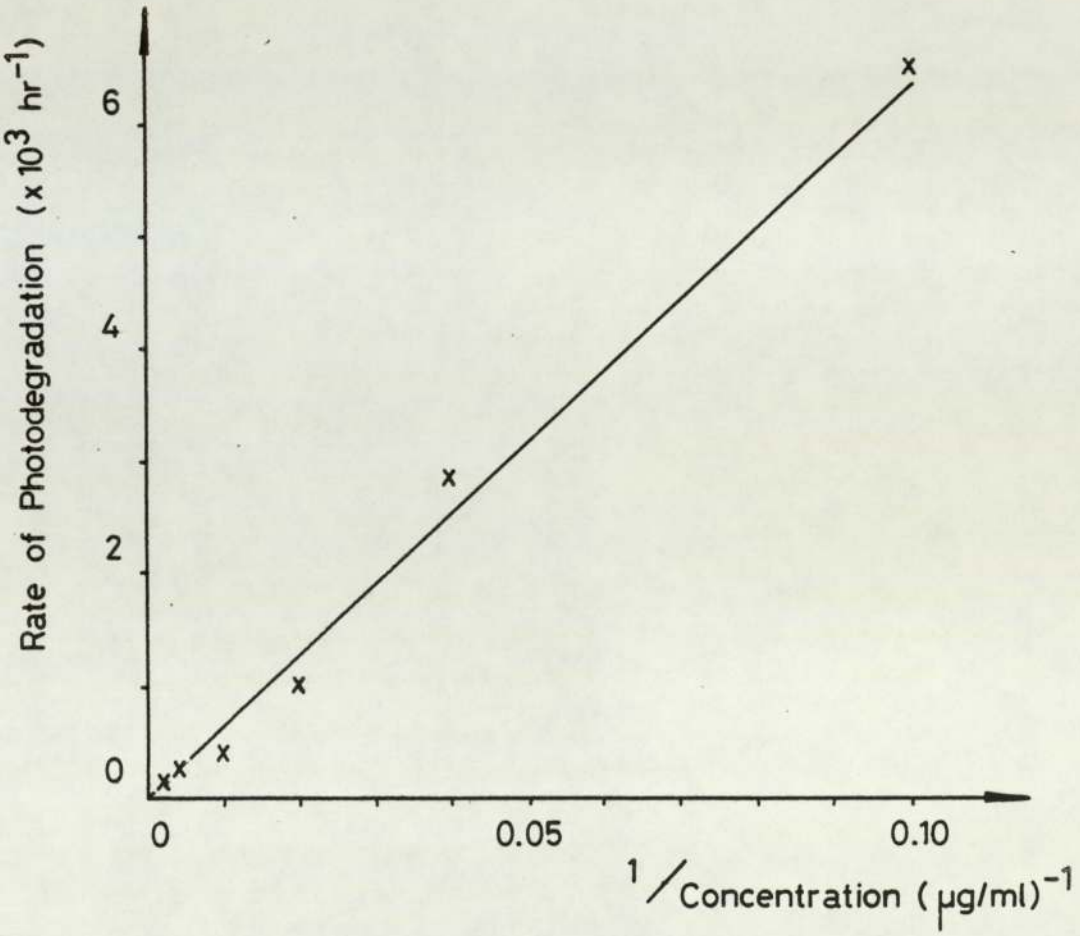


Figure 3.2. The rate constant, k , for photodegradation of doxorubicin shown as a function of the reciprocal of the initial drug concentration.

DRUG	RATE CONSTANT k (hr^{-1})			
	100µg/ml	50µg/ml	25µg/ml	10µg/ml
EPI	3.75×10^{-4}	1.41×10^{-3}	2.69×10^{-3}	5.35×10^{-3}
DAU	4.92×10^{-4}	1.23×10^{-3}	2.78×10^{-3}	5.13×10^{-3}
DOX	3.93×10^{-4}	1.01×10^{-3}	2.85×10^{-3}	6.50×10^{-3}

Table 3.3. Rate constants for photodegradation of doxorubicin, daunorubicin and epirubicin in aqueous solution between 100µg/ml and 10µg/ml (clear glass) .

DRUG	t90% values (hours)			
	100µg/ml	50µg/ml	25µg/ml	10µg/ml
EPI	> 168	86	32	13
DAU	> 168	91	30	15
DOX	> 168	88	31	12

Table 3.4. T90% values for photodegradation of aqueous solutions of doxorubicin, daunorubicin and epirubicin between 100µg/ml and 10µg/ml (clear glass).

Tables 3.3, 3.4 and 3.5 show the rate constants for photodegradation, the t90% values and the percentage of each drug remaining respectively, in clear glass, after exposure of solutions between 10µg/ml and 100µg/ml to room light for 168 hours.

Results from a two-way analysis of variance (with replicates) on the data in Table 3.5 are shown in Table 3.6. For the rows (DF 3,24), the F value from Tables [180] , at the 5% level of significance, was 3.01. As this value was much smaller than the F-ratio calculated in Table 3.6 this indicated that the difference between the mean percentage of each drug remaining in the rows was highly significant or that photodegradation was dependent on the concentration of the solution. Results earlier in this section indicated that the rate of photodegradation was proportional to the reciprocal of the drug concentration. This proportionality was due to the fact that photodegradation increased (as a proportion of the total amount) as the concentration of the drug decreased.

For the columns (DF 2,24) the F value from Tables [180], at the 5% level of significance, was 3.40. As this value was smaller than the F-ratio calculated in Table 3.6 this indicated that the difference between the mean percentage remaining in the columns was also significant which suggested a slight difference in the photodegradation behaviour of the three analogues. However, a more detailed analysis of the data which entailed a comparison of each drug firstly with one analogue and then the other, using Student's t Test, indicated that for solutions between

25μg/ml and 100μg/ml there was no significant difference between the percentage of each drug remaining at the end of the study ($p > 0.05$). The difference between the percentage of each drug remaining at 10μg/ml was significant ($p < 0.05$). These data indicate that in most situations it should be possible to predict the rate of degradation of daunorubicin and epirubicin in fluorescent light from data obtained for doxorubicin.

Results from control solutions showed that the rate of disappearance of doxorubicin in the dark (Tables 3.7.a and 3.7.b) was markedly slower than the rate of degradation of equivalent solutions which were exposed to room light (Tables 3.2.a and 3.2.b). Results from a two-way analysis of variance (with replicates) on the data in Table 3.8 are shown in Table 3.9. For the rows (DF 5,36) the F value from Tables [180], at the 5% level of significance, was 2.49 approximately. As this value was much smaller than the calculated F-ratio in Table 3.9 this indicated that the difference in the mean percentage remaining of each drug in the rows was significant or that drug loss in the dark was dependent on the concentration of the solution. This relationship is explained later in this section. For the columns (DF 2,36) the F value from Tables [180], at the 5% level of significance, was 3.28 approximately. As this value was much smaller than the calculated F-ratio in Table 3.9 this indicated that the difference between the mean percentage of doxorubicin remaining was also significant in the columns or that drug loss in the dark was affected by

the container type. A more detailed analysis of the data which entailed a comparison of each type of container (firstly the two glass containers then the clear glass and polyethylene and finally the amber glass and polyethylene), using Student's t Test, indicated that there was no significant difference between the percentage of doxorubicin remaining in any of the containers stored in the dark between 500μg/ml and 25μg/ml ($p > 0.05$). Drug loss in all cases was less than 10%. However, at 10μg/ml the amount of doxorubicin lost from the polyethylene containers was significantly smaller than from either the clear or amber glass containers ($0.01 < P < 0.001$).

A larger apparent drug loss was observed as the initial concentration of the solution decreased between 100μg/ml and 10μg/ml. As chromatographic traces showed no evidence of degradation products it was concluded that drug loss from solutions stored in the dark was probably due to adsorption onto the container.

Adsorption of doxorubicin onto glass has been documented in the literature [182,183] but this phenomenon was apparently not observed by Tavoloni et al [169]. These authors indicated that aqueous solutions of doxorubicin, stored in glass containers in the dark, showed no decrease in concentration irrespective of the initial concentration.

In summary, the data in this experiment indicate that storage of doxorubicin in aqueous solution, pH 6.1, in polyethylene containers was preferable to glass, especially at concentrations less than 25μg/ml. Although adsorption of doxorubicin to polyethylene has been observed [182], the

data in Table 3.7. indicated that for solutions greater than or equal to 10µg/ml, storage in polyethylene was acceptable for at least 168 hours.

DRUG	PERCENTAGE PARENT DRUG REMAINING ($\bar{x}$ ± SD n = 3)			
	100µg/ml	50µg/ml	25µg/ml	10µg/ml
EPI	93.0 ± 1.7	79.8 ± 1.2	62.7 ± 1.5	41.8 ± 2.2
DAU	92.7 ± 1.4	81.2 ± 1.4	61.8 ± 2.2	42.2 ± 1.1
DOX	93.3 ± 1.4	84.9 ± 0.6	61.5 ± 1.5	31.8 ± 1.4

Table 3.5. Percentage doxorubicin, daunorubicin and epirubicin remaining after exposure of an aqueous solution to room light for 168 hours (clear glass).

SOURCE	SUM SQUARES	D.F.
Rows	15505.17	3
Columns	21.6875	2
Interactions	229.5313	6
Residuals	58.0625	24
Total	15814.45	35

SOURCE	MEAN SQUARE	F-RATIO (DF1,DF2)
Rows	5168.391	2316.342 (3 , 24)
Columns	10.84375	4.482239 (2 , 24)
Interactions	38.25521	15.8127 (6 , 24)
Residuals	2.419271	

Table 3.6. Two-way analysis of variance (with replicates) for the photodegradation of doxorubicin, daunorubicin and epirubicin in clear glass.

Note: Rows = The effect of concentration.
Columns = The effect of the drug.

CONTAINER	RATE CONSTANT k (hr^{-1})		
	500µg/ml	250µg/ml	100µg/ml
CLEAR GLASS	7.06×10^{-5}	1.54×10^{-4}	1.05×10^{-4}
AMBER GLASS	1.27×10^{-4}	1.39×10^{-4}	6.60×10^{-5}
POLYETHYLENE	7.69×10^{-5}	7.67×10^{-5}	2.35×10^{-4}

Table 3.7.a. Rate constants for disappearance of doxorubicin in aqueous solution in the dark between 500µg/ml and 100µg/ml.

CONTAINER	RATE CONSTANT k (hr^{-1})		
	50µg/ml	25µg/ml	10µg/ml
CLEAR GLASS	1.25×10^{-4}	1.40×10^{-4}	3.34×10^{-4}
AMBER GLASS	1.01×10^{-4}	1.66×10^{-4}	3.78×10^{-4}
POLYETHYLENE	5.62×10^{-4}	1.21×10^{-4}	1.79×10^{-4}

Table 3.7.b. Rate constants for disappearance of doxorubicin in aqueous solution in the dark between 100µg/ml and 10µg/ml.

CONTAINER	PERCENTAGE DOXORUBICIN REMAINING ($\bar{x} \pm$ SD n = 3)					
	DRUG CONCENTRATION (µg/ml)					
	500	250	100	50	25	10
CLEAR GLASS	98.5 ± 1.1	97.8 ± 1.2	97.0 ± 1.4	98.3 ± 1.1	96.5 ± 1.9	90.9 ± 1.3
AMBER GLASS	98.1 ± 1.3	98.3 ± 1.3	98.7 ± 1.4	98.4 ± 1.1	96.6 ± 2.0	90.5 ± 1.5
POLYETHYLENE	98.9 ± 1.0	101.5 ± 1.8	102.4 ± 1.1	98.4 ± 1.4	98.1 ± 1.0	96.9 ± 1.5

Table 3.8. Percentage doxorubicin remaining in aqueous solution after storage in the dark for 168 hours.

SOURCE	SUM SQUARES	D.F.
Rows	276.75	5
Columns	89.71875	2
Interactions	64.65625	10
Residuals	68.375	36
Total	499.5	53

SOURCE	MEAN SQUARE	F-RATIO (DF1,DF2)
Rows	55.35	29.14223 (5 , 36)
Columns	44.85938	23.61883 (2 , 36)
Interactions	6.465625	3.404205 (10, 36)
Residuals	1.899306	

Table 3.9. Two-way analysis of variance (with replicates) for solutions of doxorubicin stored in the dark.

Note: Rows = The effect of concentration.
Columns = The effect of container type.

A plot of the natural logarithm of the percentage doxorubicin remaining versus time, in half strength Tris buffer (pH 7.2 and pH 8.0), after exposure to room light, (Figure 3.3) showed a very rapid first-order decay when compared to identical control solutions which were stored in the dark (Figure 3.4). The calculated rates of degradation and t90% values for these solutions are presented in Table 3.10. A comparison was made between the rate of degradation of doxorubicin in aqueous solution (pH 6.1), which is also

shown in Table 3.10, and the rate of degradation in half strength Tris buffer at pH 7.2 and pH 8.0. Results showed that photodegradation at pH 7.2 was approximately five times faster than at pH 6.1 and photodegradation at pH 8.0 was approximately forty times faster than at pH 6.1. A comparison of the rate of degradation in control solutions revealed that degradation at pH 7.2 was approximately one hundred times faster than at pH 6.1 and at pH 8.0 was about two hundred and eighty times faster than at pH 6.1.

From these data it can be concluded that both photodegradation of doxorubicin, and degradation in control solutions stored in the dark, was dependent on the pH of the solvent used. The dependence of the photodegradation of doxorubicin on the nature of the solvent has been documented in the literature. Tavoloni et al [169] observed that doxorubicin appeared to be protected from photodegradation when dissolved in fresh rat bile. This protective effect may have been due to filtration of radiation by the yellow-green colour of bile or due to stabilisation or complexation with the solvent or components of the solvent. These authors also observed that doxorubicin also became progressively less stable in ethanol, sodium chloride 0.9%, distilled water and Ringer-Krebs bicarbonate. This stability sequence was independent of the drug concentration and the intensity of light.

Tavoloni et al [169] offered no explanation for the observed differences in the rate of photodegradation in the latter four solvents and the pH of these solvents was not

recorded. If it is assumed that the pH values were within pharmacopoeial limits, this suggests that the pH of the Ringer-Krebs bicarbonate was probably greater than 7.0. As described in section 4.3.ii. and shown in Figure 4.10 doxorubicin has several ionisable functions. At pH values greater than 7 formation of the singly charged anion (with the negative charge at either the C-11 or C-6 phenolic oxygen) may occur. The structure of the C-9 side-chain present in doxorubicin also favours enolisation and ionisation in weakly alkaline solution to form the negatively charged enolate anion as shown in Figure 4.16.

In summary, these data suggest that anion formation is promoted in weakly alkaline solution. It is possible that these anions may be more susceptible to oxidation which has been observed to occur during photodegradation and is described later in this section. Therefore, it is possible that increased oxidation may account for the higher rate of photodegradation observed by Tavoloni et al [169] in Ringer-Krebs bicarbonate.

Chromatograms obtained for aqueous solutions of doxorubicin, daunorubicin and epirubicin (10µg/ml) in clear glass, which were exposed to room light, showed a decrease in concentration of the parent compound with time. Figure 3.5. shows the chromatogram obtained for doxorubicin after exposure to room light for 168 hours. In addition to the parent drug peak, three other peaks, which eluted close to the solvent front (retention times 1.2, 1.4 and 1.6 minutes), were also observed. Similar peaks were noted on traces obtained for epirubicin and daunorubicin in clear

glass and for doxorubicin in polyethylene containers. As there was no evidence of these peaks on traces obtained for solutions stored in amber glass and solutions stored in the dark this suggested that these peaks were indicative of photodegradation products.

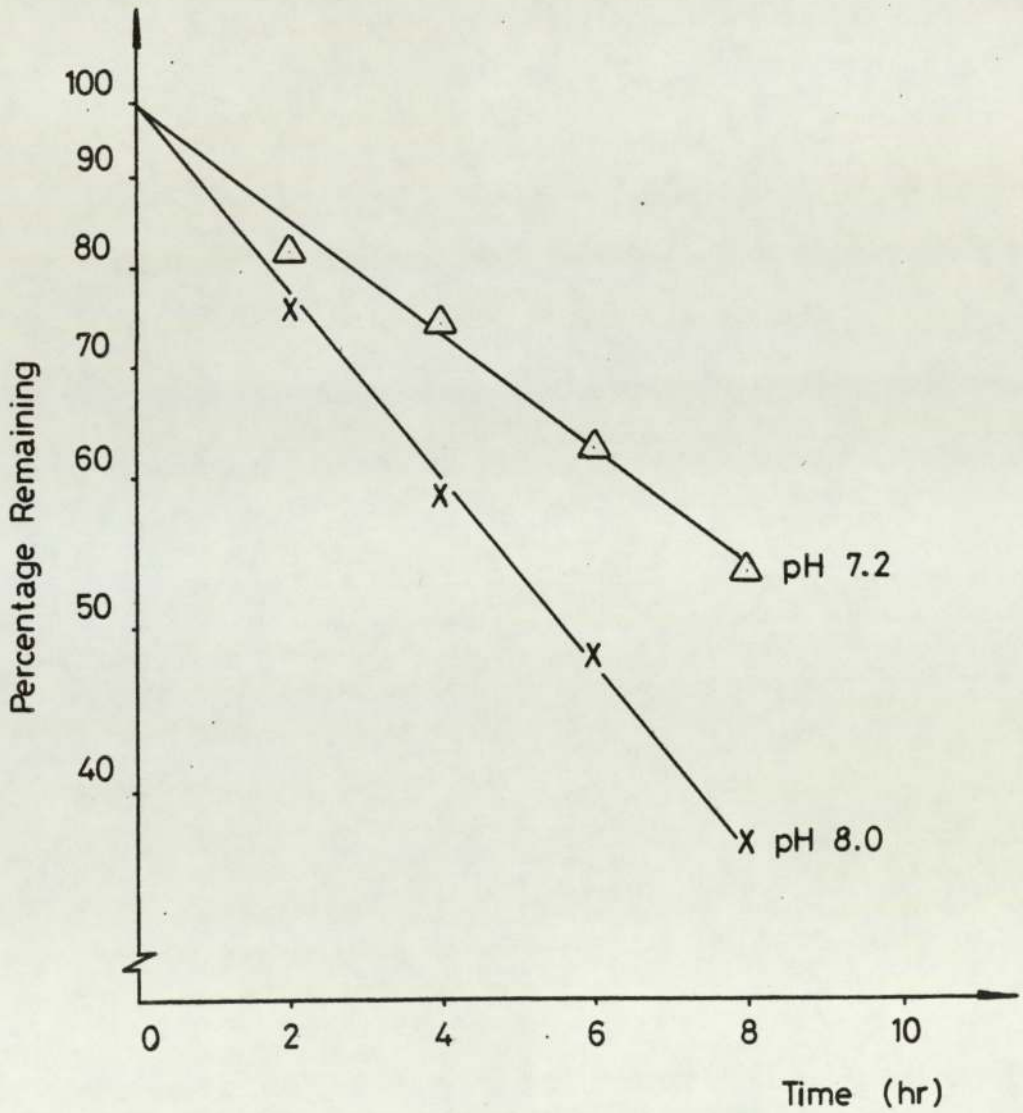


Figure 3.3. Natural logarithm of the percentage doxorubicin remaining versus time in half strength Tris buffer (pH 7.2 and 8.0) in room light (clear glass).

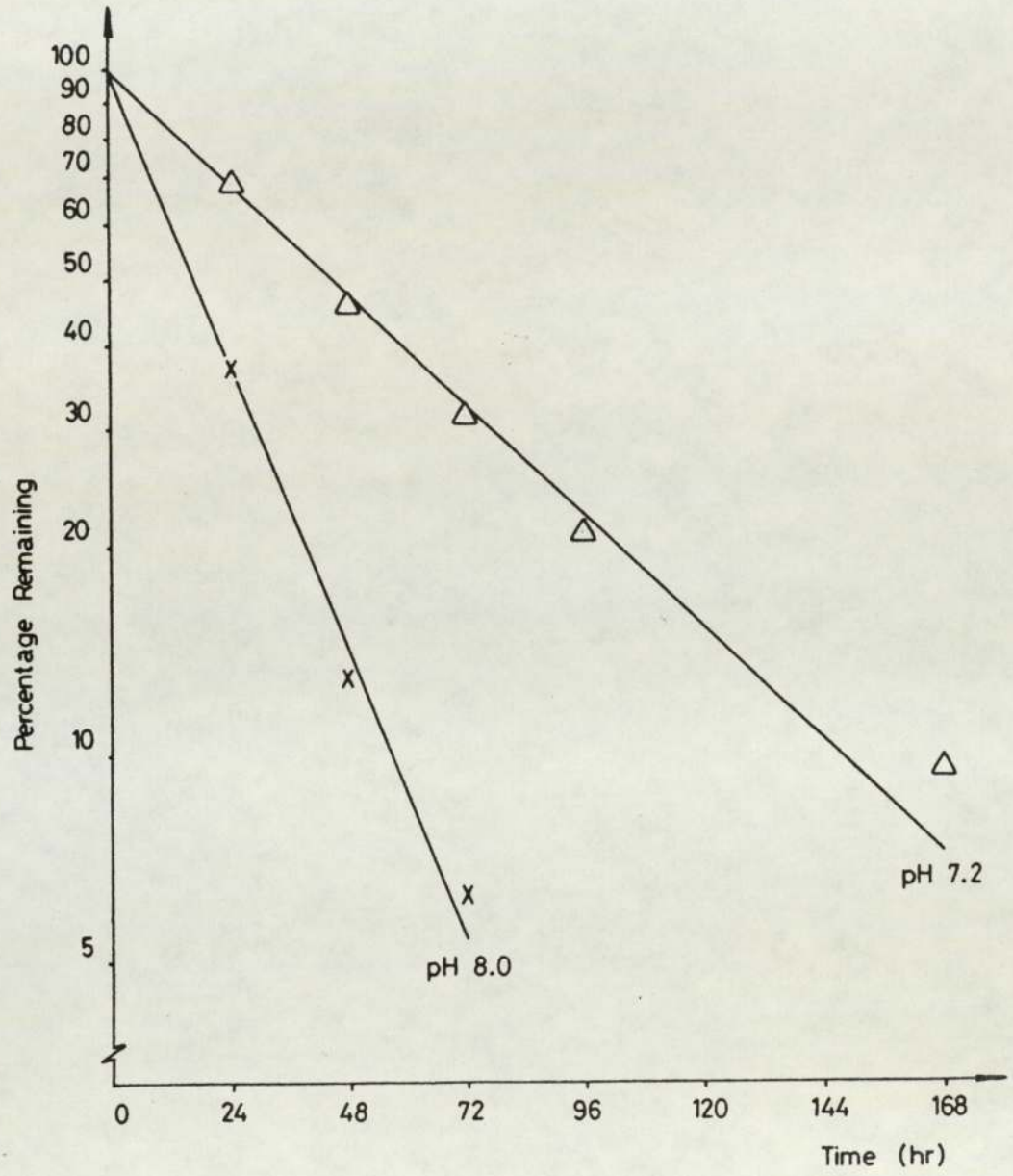


Figure 3.4. Natural logarithm of the percentage doxorubicin remaining versus time in half strength Tris buffer (pH 7.2 and pH 8.0) in the dark (clear glass).

pH OF ADMIXTURE	RATE CONSTANT (hr^{-1})	t90% VALUE (hrs)
6.1 (room light)	2.85×10^{-3}	32
6.1 (dark)	1.40×10^{-4}	> 168
7.2 (room light)	7.65×10^{-2}	1.30
7.2 (dark)	1.41×10^{-2}	6.00
8.0 (room light)	1.23×10^{-1}	0.80
8.0 (dark)	3.90×10^{-2}	1.80

Table 3.10. Rate constants and t90% values for degradation of doxorubicin (25μg/ml) in half strength Tris buffer at pH 7.2 and pH 8.0 (clear glass).

Williams and Tritton [167] found no evidence of degradation products after HPLC assay of irradiated solutions of doxorubicin. Gel filtration studies, by those authors, showed formation of a new higher molecular weight species, postulated to be a polymer containing approximately ten monomeric units, which eluted with the void volume of the column (Figure 3.6). Polymer formation would also not be detectable by HPLC as these high molecular weight species would probably not be eluted from the column.

Another possibility is that aglycones may be produced during photodegradation. Aglycones would be expected to elute after the parent compound due to their low polarity.

It is unlikely that the peaks shown in Figure 3.5 were due to aglycone formation as they eluted shortly after the void volume of the column.

Chromatograms obtained for solutions of doxorubicin, 25μg/ml, in half strength Tris buffer, pH 7.2 and pH 8.0, which were exposed to room light, (Figure 3.7) showed the following results. After two and eight hours exposure to light, solutions at pH 8.0 and pH 7.2 respectively, each showed one broad peak (DVIII) which eluted after doxorubicin. Literature data suggested that this peak was indicative of aglycone formation. If this were the case the aglycones would be expected to form a precipitate as photodegradation proceeded due to their inherently poor solubility in aqueous media. However, more detailed studies of the degradation of doxorubicin in alkaline solution which were recently published by Beijnen et al [184] and results in the following chapter suggested that precipitate formation was dependent upon several factors including the drug concentration but was not expected at concentrations less than or equal to 25μg/ml.

Chromatograms obtained after 96 hours and 48 hours exposure to room light, at pH 7.2 and 8.0 respectively, showed almost total disappearance of both the parent compound and aglycone peaks (Figure 3.8). These traces showed peaks, which eluted close to the solvent front, which were similar to those observed after photodegradation of aqueous solutions of doxorubicin (Figure 3.5). This suggested that in weakly alkaline solution aglycone formation probably occurred in the initial stages of

photodegradation but that further exposure to light resulted in the breakdown of these aglycones to other products. The reasons for breakdown of the aglycones and a possible mechanism for this reaction is discussed later in this section.

Chromatograms obtained from control solutions, 25µg/ml in Tris buffer, which were stored in the dark, also showed evidence of aglycone formation (Figure 3.9). Analysis of the full degradation profile of these solutions was not possible due to a lack of time.

A review of the literature revealed two studies which had investigated the photodegradation of doxorubicin in alkaline buffer solutions. The first of these studies [168] found that a solution of doxorubicin, 88µg/ml in phosphate buffer, pH 8.0, degraded to 34% of the original concentration after 30 minutes exposure to white light (150W) when placed 90cm away from the sample. These authors offered no further data on this degradation as they were concerned with the anti-bacterial and *in vitro* deoxyribose damaging activity of doxorubicin rather than the kinetics of degradation.

In the second study, [166] a solution of daunorubicin, in 50mM sodium chloride with 50mM phosphate buffer, pH 7.05, was irradiated and a precipitate was produced. Reversed-phase HPLC assay of this precipitate (Figure 3.10), with a mobile phase of methanol:water (97:3 vol:vol), showed five degradation products which were postulated to be aglycones. One of these aglycones was positively identified as

daunorubicinone but the major insoluble photoadduct was 7,8-dehydro-9,10-desacetyldaunorubicinone (DrVIII) which is shown in Figure 4.14. The degradation scheme for conversion of doxorubicin into DrVIII, in weakly alkaline solution, is shown in Figure 3.11. Gray and Phillips [166] also postulated that the precipitate was further degraded by a photobleaching process. The possibility that a direct photobleaching process could occur without proceeding via an aglycone was also considered, although mechanisms for these processes were not suggested by these authors.

Gray and Phillips [166] also compared the photodegradation of daunorubicin, at pH 7.05, in the presence of oxygen, with photodegradation in an atmosphere of argon. Those authors observed that photodegradation was accelerated in the presence of oxygen and that the photobleaching process described above was oxygen dependent. Similarly, Beijnen et al [185] observed that the yield of DrVI and DrVIII (Figure 4.14) formed as a precipitate during photodegradation of daunorubicin, at pH 8.0, was decreased in the presence of oxygen due to its subsequent oxidation. These authors also observed that continued oxygen-catalysed degradation led to a complete discolouration of the solution and the disappearance of DrVI and DrVIII. For DrVI it has been established that oxidising agents can break the chromophore into smaller fragments such as 3-methoxy-phthallic acid and trimellitic acid [186]. Mechanisms for the oxidation of doxorubicin and the fragmentation of the anthraquinone ring system are shown in Figures 3.12 and 3.13 respectively.

The above data indicate that the disappearance of aglycone degradation products after prolonged exposure of doxorubicin to light in the Tris buffer study was probably due to photobleaching of the aglycone (Figure 3.8) which was enhanced by the presence of oxygen in contact with the solutions in this experiment. The influence of oxygen could be eliminated by freshly boiling and cooling the water used for preparation of the drug solutions and if buffers were used these could be purged with nitrogen. Beijnen et al [185] also observed that traces of metal ion impurities, which may have originated from the buffers, also catalysed the decomposition. This effect could be prevented by addition of the chelating agent sodium edetate.

In another study Tavoloni et al [169] observed that solutions of doxorubicin did not degrade when stored in the dark. The pH of the Ringer-Krebs bicarbonate solution, which was one of the solvents used by these authors, should have been approximately 7.0. If this assumption is correct the results of the Tris buffer study and the results of Gray and Phillips [166], predict that aglycones were likely to be produced in that solution. Tavoloni et al [169] used fluorescence assay as the method of drug quantitation which does not differentiate between parent compounds and aglycones [46] therefore, some of the data presented by those authors may be inaccurate. For this reason, kinetic data, which were obtained by HPLC assay, in the above experiments, are more likely to present an accurate picture of the photodegradation of doxorubicin.

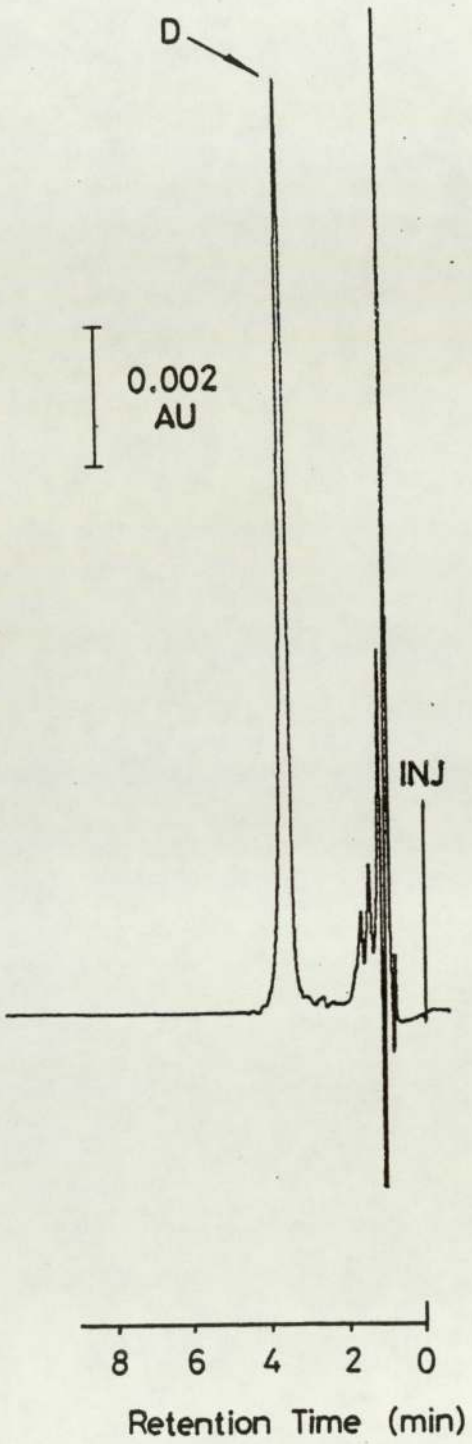


Figure. 3.5. High performance liquid chromatogram obtained after 70% photodegradation of doxorubicin (10µg/ml in clear glass at 168 hours). Mobile phase: acetonitrile:water (40:60 vol:vol; pH 2.5), flow rate: 1.4ml/min, detection: 290nm, chart speed: 5mm/min., sensitivity: 0.02 AUFS, doxorubicin = D.

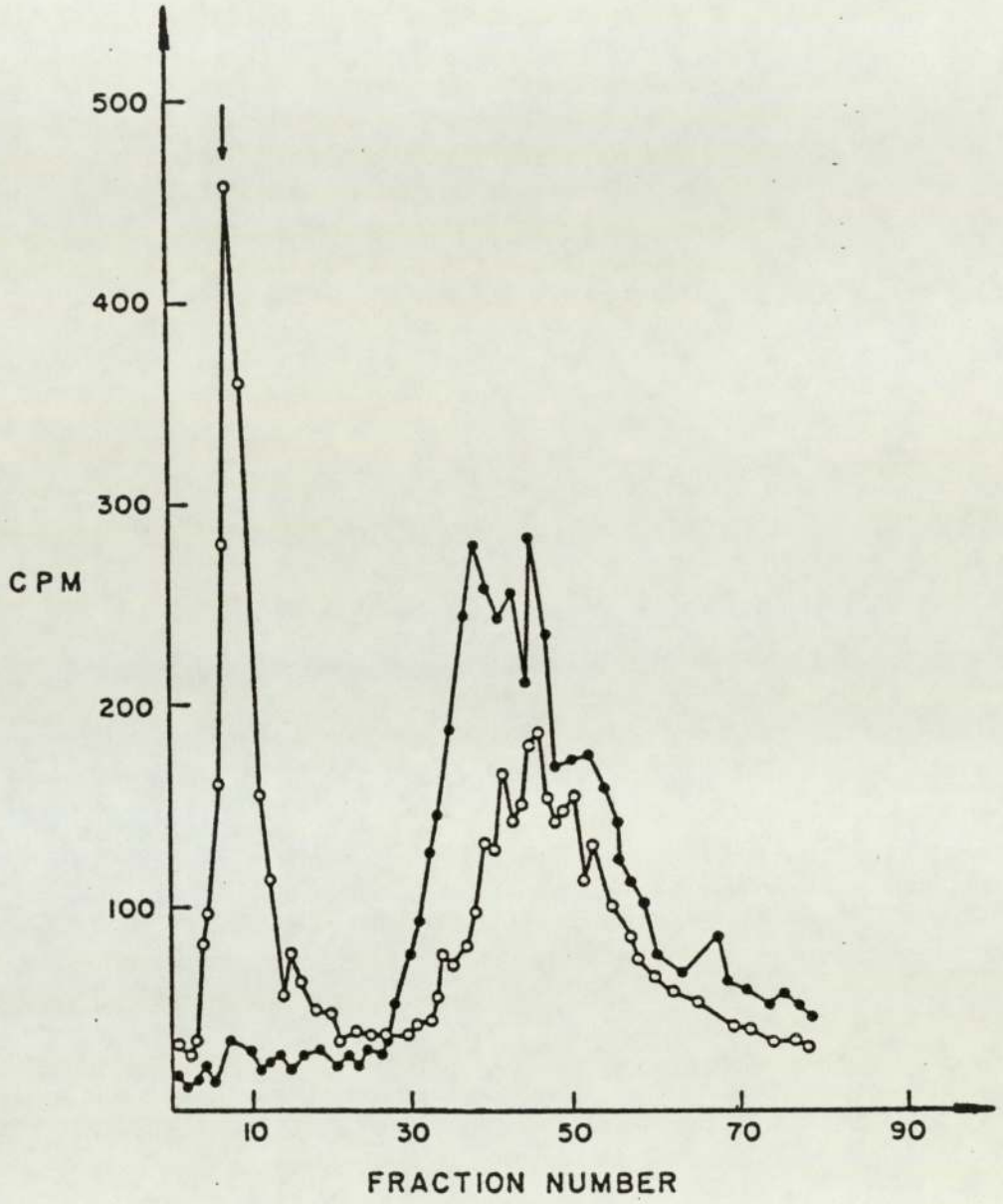
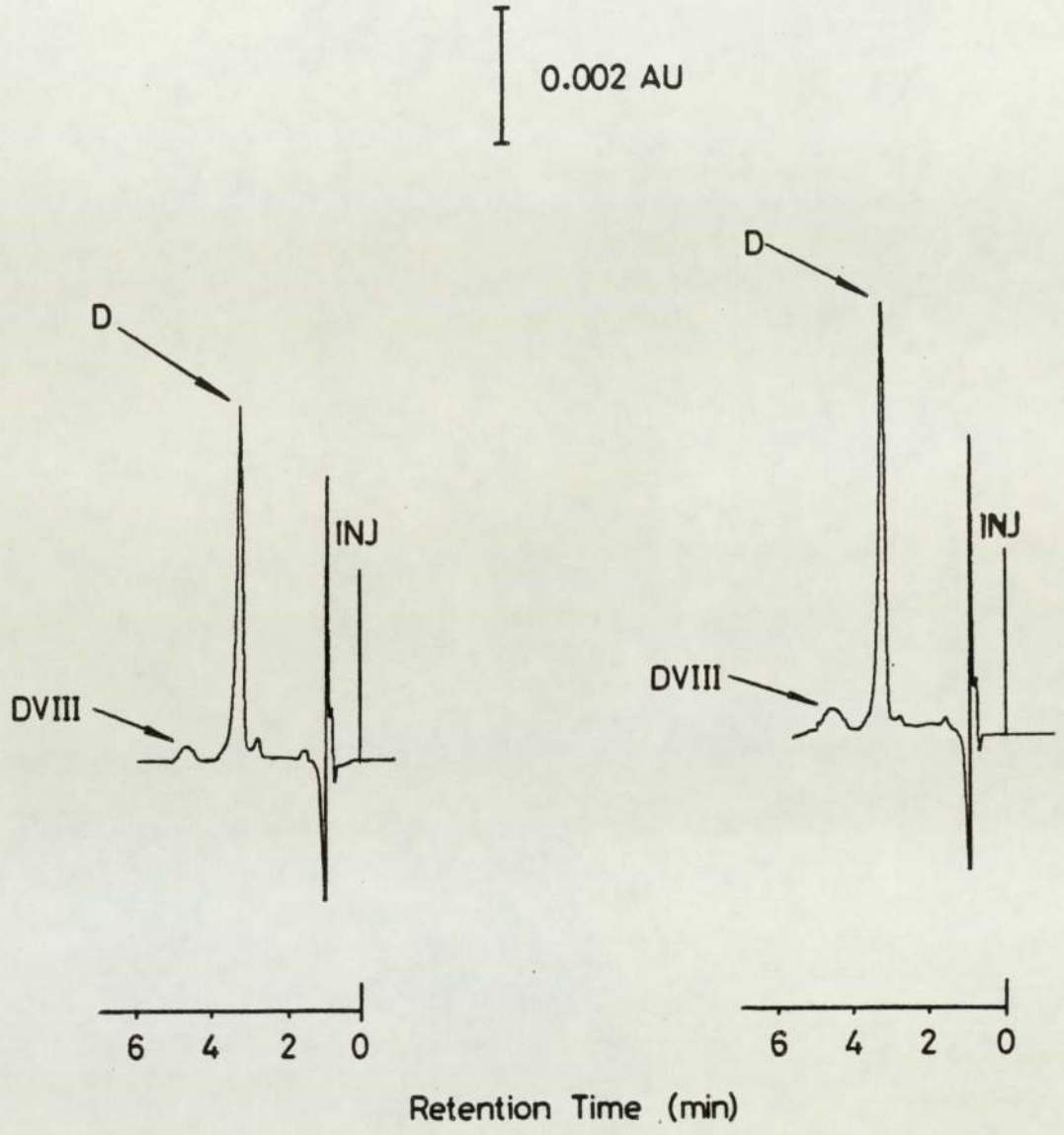


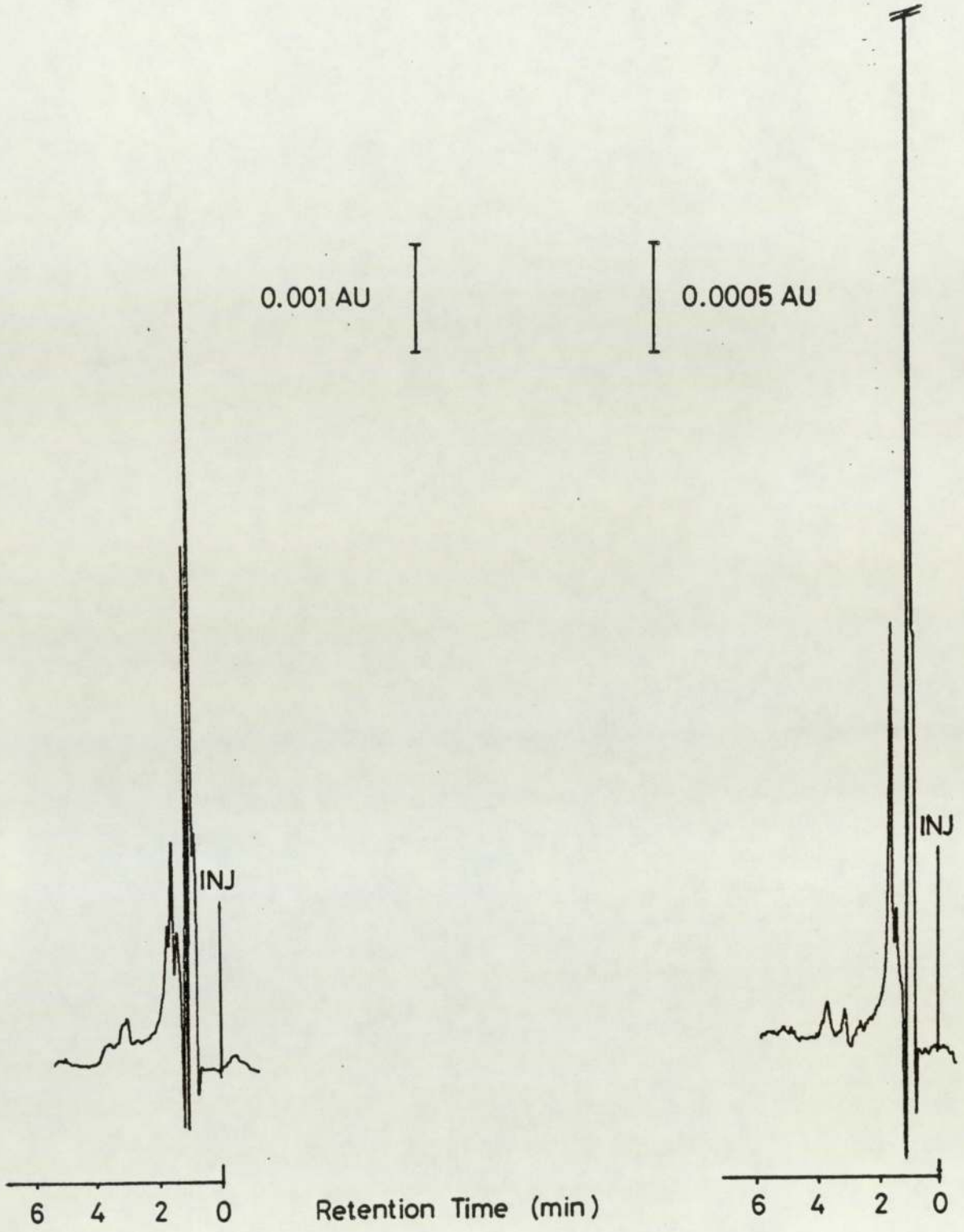
Figure 3.6. Elution of control, unirradiated (●) and 6 hour irradiated (O) [^{14}C]-adriamycin from a 0.7 x 30cm Biogel P-2 column. The arrow marks the void volume (from Williams and Tritton [167].



a) pH 7.2 after 8 hours exposure to light.

b) pH 8.0 after 2 hours exposure to light.

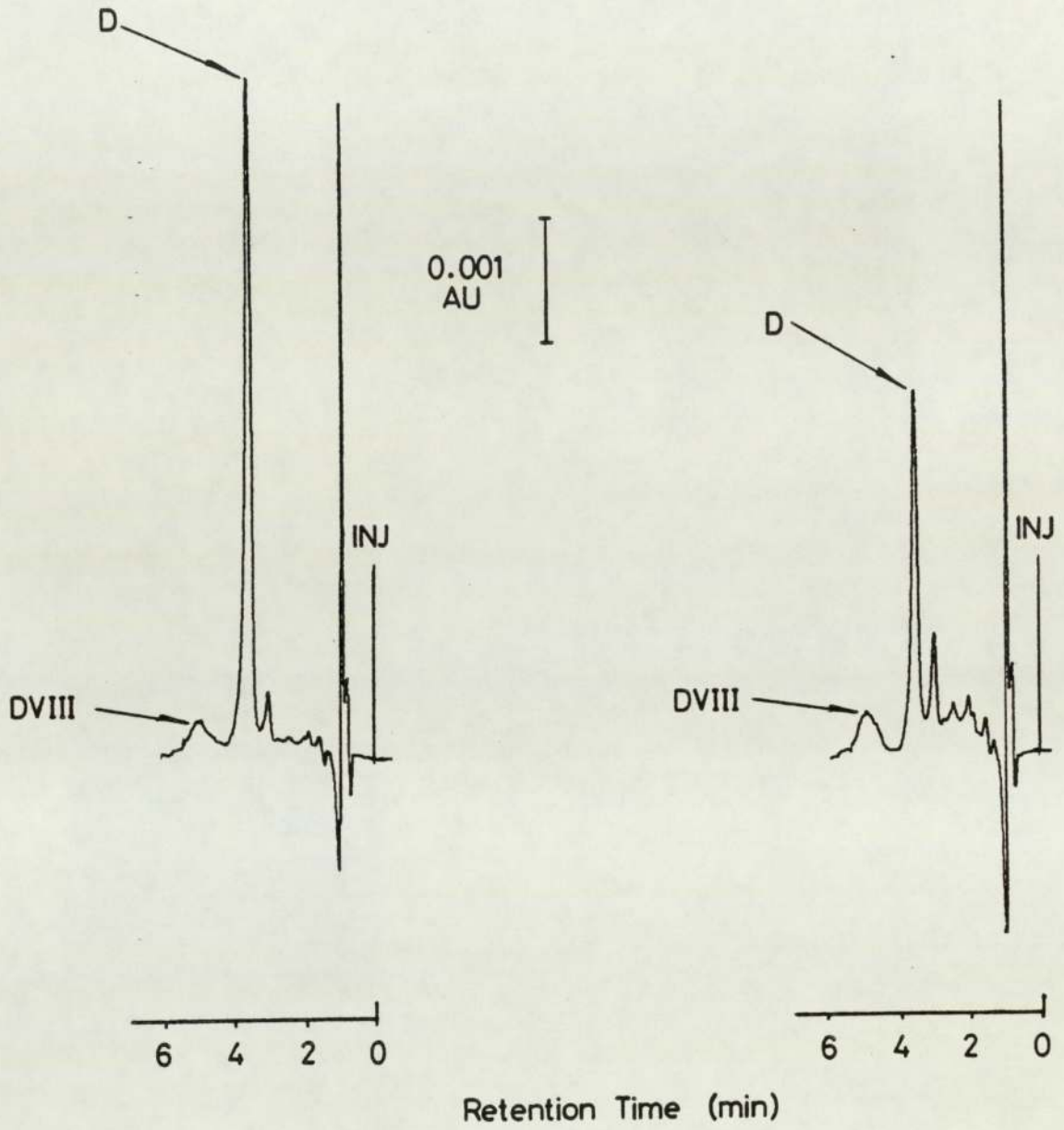
Figure 3.7. High performance liquid chromatograms obtained after exposure of solutions of doxorubicin (25µg/ml) in half strength Tris buffer (pH 7.2 and pH 8.0) to room light for 8 hours and 2 hours respectively. Mobile phase: acetonitrile: water (40:60 vol:vol; pH 2.5), flow rate 1.4ml/min, detection: 290nm, chart speed: 5mm/min, 0.02 AUFS, doxorubicin = D, aglycone degradation product = DVIII.



a) pH 7.2 after 96 hours exposure to light. 0.01 AUFS

b) pH 8.0 after 48 hours exposure to light. 0.005 AUFS

Figure 3.8. High performance liquid chromatogram obtained after exposure of solutions of doxorubicin (25µg/ml) in half strength Tris buffer (pH 7.2 and pH 8.0) to room light for 96 hours and 48 hours respectively. Mobile phase: acetonitrile :water (40:60 vol:vol; pH 2.5), flow rate: 1.4 ml/min., detection: 290nm, chart speed: 5mm/min..



a) pH 7.2

b) pH 8.0

Figure 3.9. High performance liquid chromatograms obtained for the degradation of doxorubicin (25µg/ml) in half strength Tris buffer (pH 7.2 and pH 8.0) after storage in the dark for 24 hours. Mobile phase: acetonitrile:water (40:60 vol:vol; pH 2.5), flow rate; 1.4ml/min, detection: 290nm, chart speed: 5mm/min, 0.01 AUFS, doxorubicin = D, aglycone degradation product = DVIII.

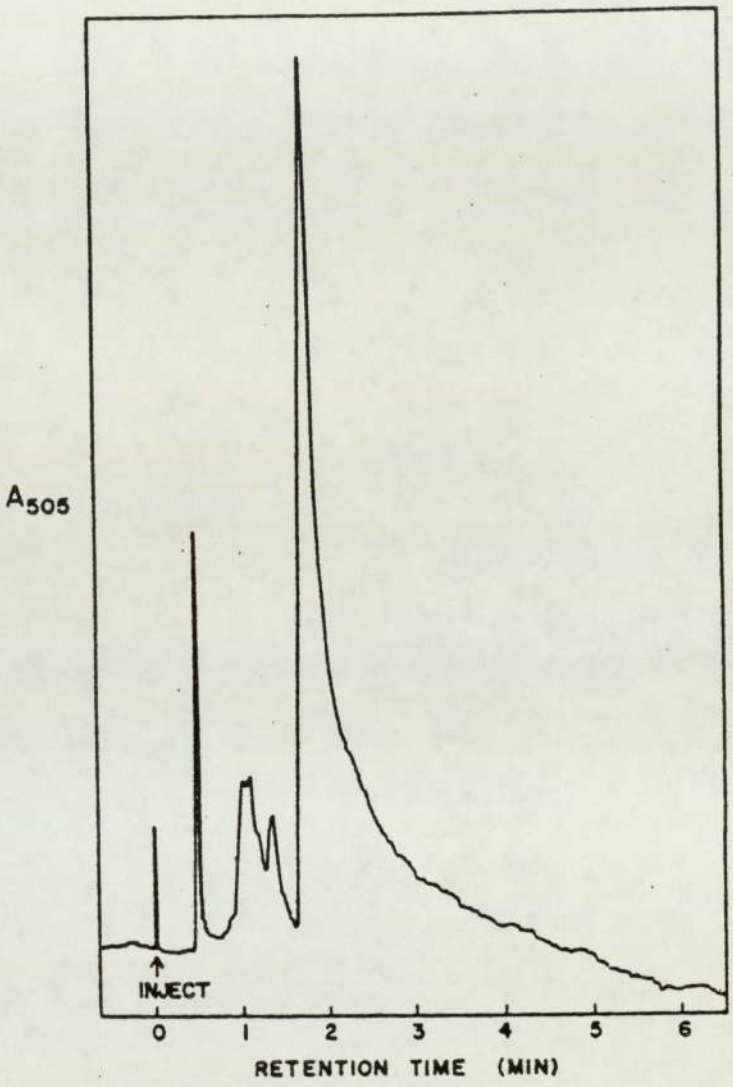
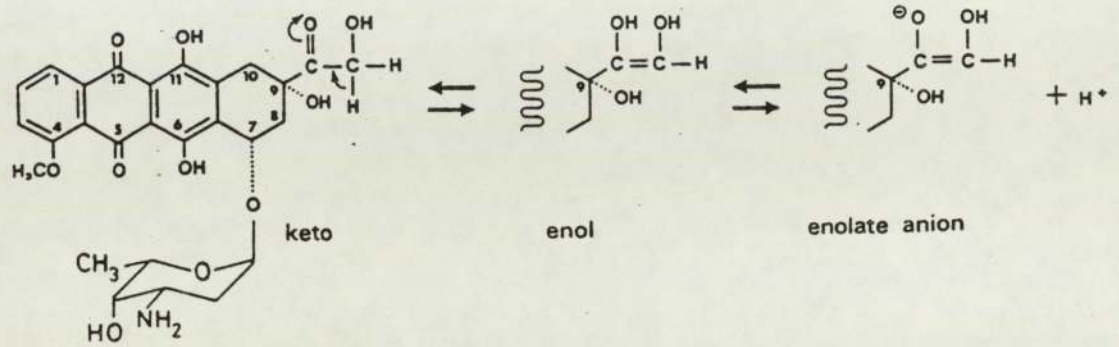
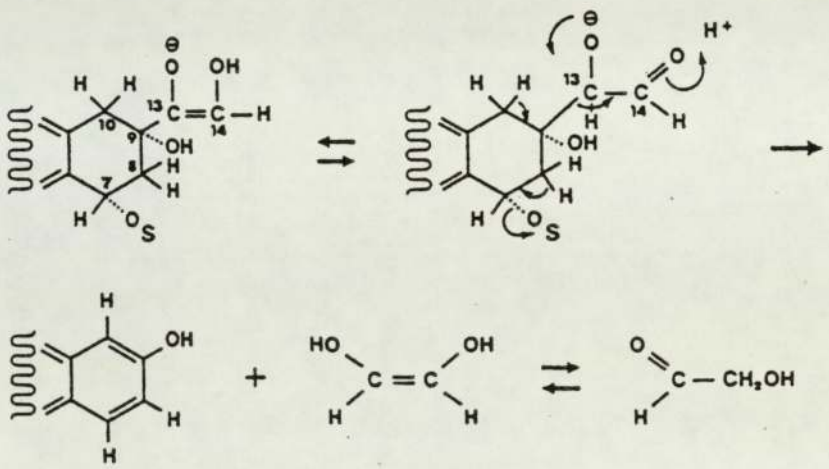


Figure 3.10. High performance liquid chromatogram of the precipitate obtained after ten minutes UV irradiation of daunorubicin (540μM) in the presence of Argon. The solvent was 50mM sodium chloride, 50mM phosphate buffer, pH 7.05. Mobile phase: methanol:water (97:3 vol:vol), flow rate 3.5ml/min, detection 505nm (from Gray and Phillips [166]).





DVIII

Figure 3.11. Degradation scheme for conversion of doxorubicin into 7,8-dehydro-9,10-desacetyldaunorubicinone (DVIII) (from Beijnen et al [184]).

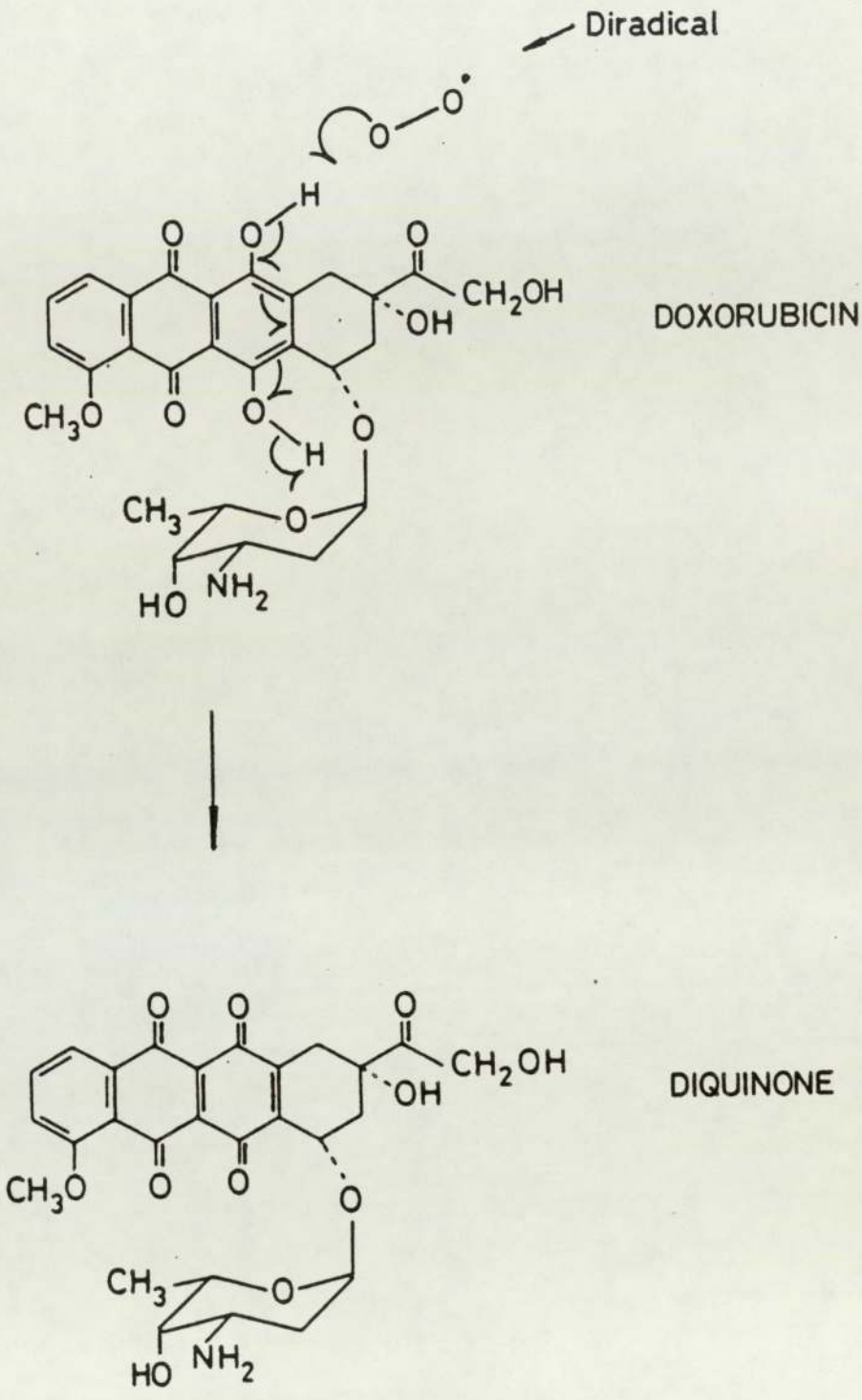


Figure 3.12. Postulated scheme for the oxidation of doxorubicin to the diquinone.

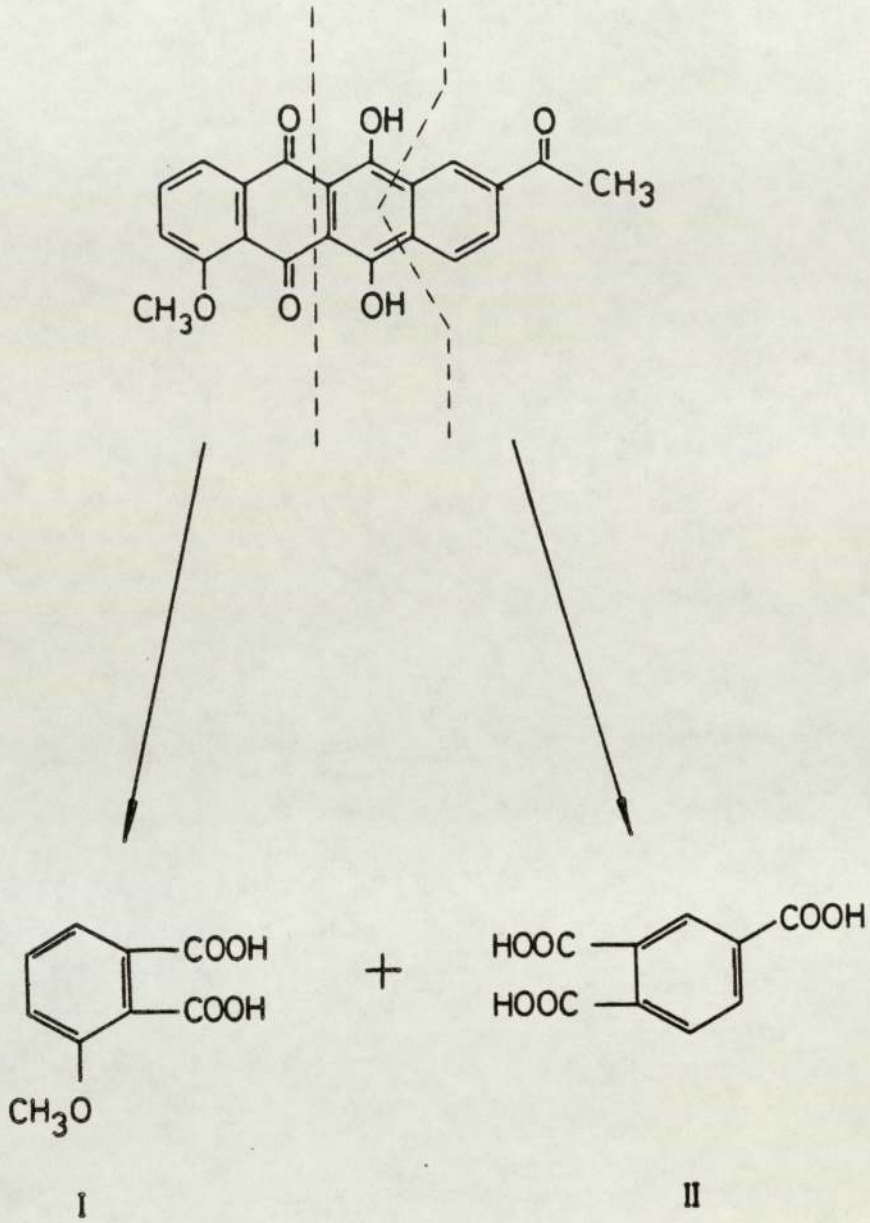


Figure 3.13. Mechanism for conversion of 7,8,9-bisanhydrodaunorubicinone to 3-methoxyphthallic acid (I) and trimellitic acid (II) (from Arcamone et al [186]).

The data from the preceeding experiments show that aglycones were produced in solutions of doxorubicin in Tris buffer, pH 7.2 and pH 8.0, both after exposure to light and after storage in the dark. This indicates that aglycones were not solely photodegradation products as postulated by Gray and Phillips [166], but were formed as a result of the particular mechanism of degradation at these pH values. A more complete study of the effects of the pH of the solvent on the rate and mechanism of degradation is presented in the following chapter.

At concentrations such as those used for cancer chemotherapy (2mg/ml) no special precautions are necessary to protect freshly prepared solutions of these agents from light. However, data have been published which indicate that when a solution of doxorubicin (500µg/ml) was irradiated with UV light, at 366nm, a new higher molecular weight species formed, postulated to be a polymer consisting of about ten monomeric units, that could not penetrate the cell. The net result of this polymer formation was that the drug lost its ability to be cytotoxic to Sarcoma 180 cells *in vitro.* [167]. These authors also observed that doxorubicin was similarly inactivated under fluorescent light in the laboratory. These data suggest that solutions of anthracyclines for therapeutic use should not be exposed to fluorescent light or sunlight for prolonged periods.

The results of this study are of particular importance when these drugs are handled in low concentrations in the laboratory. At concentrations below 100µg/ml decomposition may be significant if the drugs are exposed to fluorescent

light for sufficient time.

Other authors have observed that photodegradation is directly proportional to the intensity of the light and the distance of the samples away from the source [169]. In this study the room light, which was provided by four 65/80 watt ceiling fluorescent tubes mounted approximately one metre above the samples, was considered to be of a relatively low intensity and consequently the t90% values for doxorubicin, daunorubicin and epirubicin (in clear glass at 10μg/ml) were were relatively high (12 hours, 15 hours and 13 hours respectively). These data suggest that drug loss during photodegradation (for solutions in the low nanomolar range) may become extremely rapid if the solutions are close to high intensity fluorescent light or sunlight.

Photodegradation was most rapid in clear glass containers compared to amber glass and opaque polyethylene which appeared to afford some protection from light. The difference in the rate of photodegradation observed in these containers may be related to differences in their UV transmission curves. Transmission through clear glass was greater than 90% between 750nm and 360nm, decreased sharply between 360 and 280nm and was negligible between 280 and 200nm. The amber glass containers conformed with the United States Pharmacopoeia (USP) recommendations which state that transmittance should be less than 12% between 290 and 450nm therefore these containers gave adequate protection from light. The spectrum obtained for the opaque polyethylene containers showed a gradual decrease in transmittance from

40% at 750nm to 10% at 280nm. These data suggest that some, but not complete, protection from light was afforded by these containers. In summary, photodegradation was prevented either by storage in amber glass or by covering clear glass or polyethylene containers with aluminium foil.

Other authors have also observed that photodegradation is enhanced by the presence of oxygen in contact with the solution [166,185]. The influence of oxygen can be eliminated by freshly boiling and cooling the water which is used for preparation of the solutions and if buffers are used these may be purged with nitrogen. Trace metal ion impurities, which may originate from buffers, have also been observed to catalyse decomposition [185]. This effect could be prevented by addition of the chelating agent sodium edetate.

Chromatograms from containers stored in the dark showed a decrease in the concentration of the parent compound with time. As these traces showed no evidence of degradation products this drug loss was concluded to be due to adsorption onto the container. Drug loss due to adsorption is also extremely important (as a proportion of the total amount of drug present) in solutions of very low concentration. Results from this experiment showed that adsorptive losses may be minimised by storage of these solutions in polyethylene or polypropylene containers.

Chapter Four

The kinetics of degradation of doxorubicin, daunorubicin and epirubicin.

4.1. Introduction.

The aim of the following studies was to investigate the effect of pH on the mechanism and rate of degradation of doxorubicin, daunorubicin and epirubicin by construction of a pH profile for each drug. The effects of temperature and buffer type on the rate of degradation were also investigated. Finally the use of co-solvents to prevent precipitation during degradation in alkaline buffers was studied.

4.2. Experimental.

Stock solutions of each drug were freshly prepared (section 2.2.i.) and diluted to produce standard solutions (200µg/ml). These standard solutions were used, in combination with either Britton-Robinson buffer (Appendix I) pH 1.81 to 11.98, Sorenson buffer (Appendix II) pH 1.2 to 5.0, or Tris buffer (Appendix III) pH 7.2 to 9.0 to construct a pH profile for each drug.

A 10ml aliquot of standard solution was mixed with an equal volume of buffer to yield a final concentration of 100µg/ml of drug in half strength buffer. Duplicates of each solution were stored in 20ml amber glass bottles. These bottles were sealed with plastic screw caps, covered with aluminium foil and stored at ambient temperature (normally 25°C) in a darkened room.

Sampling times were individualised for each pH value according to the observed rate of degradation in a pilot study. The following sampling protocol was adopted. Samples (2ml aliquots) were taken immediately after combination and mixing of drug and buffer solutions, then at 24 hour intervals over 168 hours for solutions between pH 1.2 and 9.0. For solutions with pH values greater than 9.0 samples were taken initially, after 30 minutes, then hourly over a period of 8 hours.

Gradual formation of a precipitate, which contained both parent drug and degradation products, in alkaline buffer admixtures, necessitated selection of a co-solvent in order that degradation kinetics could be more accurately determined. Vials of drug were reconstituted with the co-solvent:water admixture (dimethylformamide (DMF):water 50:50 vol:vol), diluted with this co-solvent:water admixture to produce standard solutions and combined with buffer as previously described. All admixtures were observed for evidence of precipitation prior to sampling to assess the suitability of each co-solvent tested. The effect of drug and buffer concentration on precipitation were also investigated. Standard solutions of doxorubicin were combined with Britton-Robinson buffer (pH 7.96 and 9.91), as previously described, to yield final drug concentrations of 100, 50 and 25μg/ml in half, third and quarter strength buffer. The effect of the co-solvent itself on the rate of degradation was studied by comparison of the rate of degradation in aqueous Sorenson buffer (pH 1.2 to 5.0) with that observed in identical drug buffer admixtures which

contained the selected co-solvent.

The effect of increased temperature on the rate of degradation of doxorubicin was studied in Sorenson buffer, pH 1.4, and Tris buffer pH 8.5. The buffer was pre-heated to the experimental temperature prior to the addition of the drug solution. After thorough mixing the flasks were stoppered, covered with aluminium foil, and placed in a water bath at either 50, 60, 70 or 80°C.

Samples (2ml aliquots) were removed initially, after 5,10,15,30,45 and 60 minutes, then at half hourly intervals over a period of four hours to assess the ideal sampling times for each temperature studied. The following sampling protocol was adopted: At 50°C and 60°C samples were taken initially then at half hourly intervals over approximately four hours. At 70°C and 80°C samples were taken at 10 minute intervals over approximately one hour. All samples were placed in an ice bath immediately after collection in order to quench the reaction. In addition, an equal amount of 0.1M hydrochloric acid was added to all samples with a pH greater than 7.0 to quench the reaction. Samples were frozen and stored at -20°C until assay (section 2.2.i.). All solutions were thawed at ambient temperature and protected from light with aluminium foil prior to assay. Duplicates of each sample were injected onto the column and eluted with the mobile phases defined in section 2.2.ii.

Chromatograms were inspected for evidence of degradation under all conditions tested. Concentrations remaining were determined by interpolation of peak areas

(section 2.3.ii.) on calibration curves which were constructed daily. All concentrations remaining, which were expressed as a percentage of the initial concentration at time zero, represented the mean of duplicate values.

Data were evaluated for first order kinetics by construction of plots of the natural logarithm of the percentage remaining versus time according to the equation;

$$\ln x_t = \ln x_o - k_{obs} . t$$

where x_t = the concentration remaining at time t

x_o = the initial concentration

k_{obs} = the observed rate constant

4.3. Results and discussion.

Decomposition of doxorubicin and daunorubicin in acidic solution has been investigated by several authors [158,159]. Prior to initiation of this study there were very few data published on the degradation of these drugs in alkaline solution. Although several authors noted the presence of degradation products these were not identified, except in the study of Abdeen [163]. Shortly after completion of the following studies data were published which elucidated the mechanism of degradation of doxorubicin and daunorubicin in alkaline solution where Beijnen et al [184,185] have

postulated structures for the degradation products of daunorubicin in acidic and alkaline solution. The results presented below are discussed in accordance with the current literature.

4.3.i. Degradation in acidic media.

Plots of the natural logarithm of the percentage parent drug remaining versus time were linear (r=0.999) which indicated that degradation in acidic solution followed first order kinetics. Figure 4.1 shows a typical first order plot for epirubicin in Britton-Robinson buffer pH 1.81 and pH 4.35. The rate constants, k, for degradation in aqueous Britton-Robinson buffer are shown in Table 4.1. These data show that epirubicin, doxorubicin and daunorubicin were optimally stable at pH 4.35. The rate of degradation of all three analogues increased as the pH of the buffer decreased between pH 4.35 and 1.81. Over this pH range doxorubicin and daunorubicin appeared to degrade at almost the same rate whereas the rate of degradation of epirubicin was substantially different. The data indicate that epirubicin was more stable than the other two analogues in acidic solution. This increased stability is clearly demonstrated in Figure 4.2 which shows the first order plot for accelerated degradation of all three analogues in Sorenson buffer, pH 1.4, at 70°C.

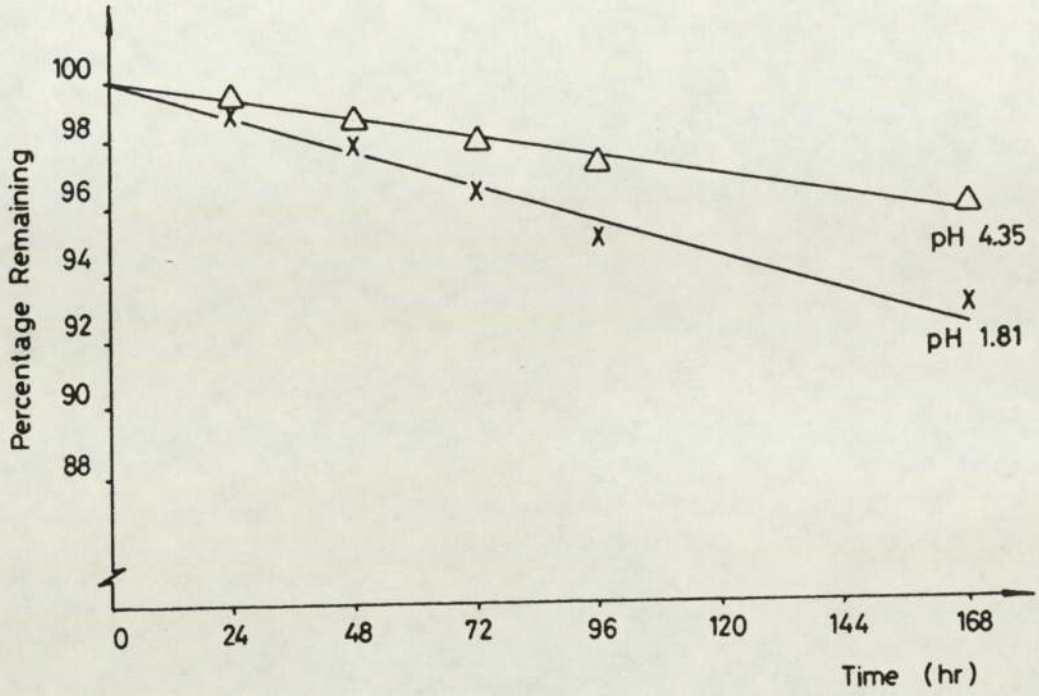


Figure 4.1. Natural logarithm of the percentage epirubicin remaining versus time in half strength Britton-Robinson buffer pH 1.81 and pH 4.35 at 25°C.

pH	RATE CONSTANT k (hr^{-1})		
	DOX	DAU	EPI
1.81	7.29×10^{-4}	7.14×10^{-4}	4.71×10^{-4}
1.98	5.57×10^{-4}	5.46×10^{-4}	4.29×10^{-4}
3.29	3.27×10^{-4}	3.48×10^{-4}	2.86×10^{-4}
4.35	3.05×10^{-4}	3.06×10^{-4}	2.47×10^{-4}
5.33	5.88×10^{-4}	4.15×10^{-4}	4.65×10^{-4}

Table 4.1. Rate constants for degradation of doxorubicin, daunorubicin and epirubicin in half strength Britton-Robinson buffer (pH 1.81 to 5.33) at 25°C.

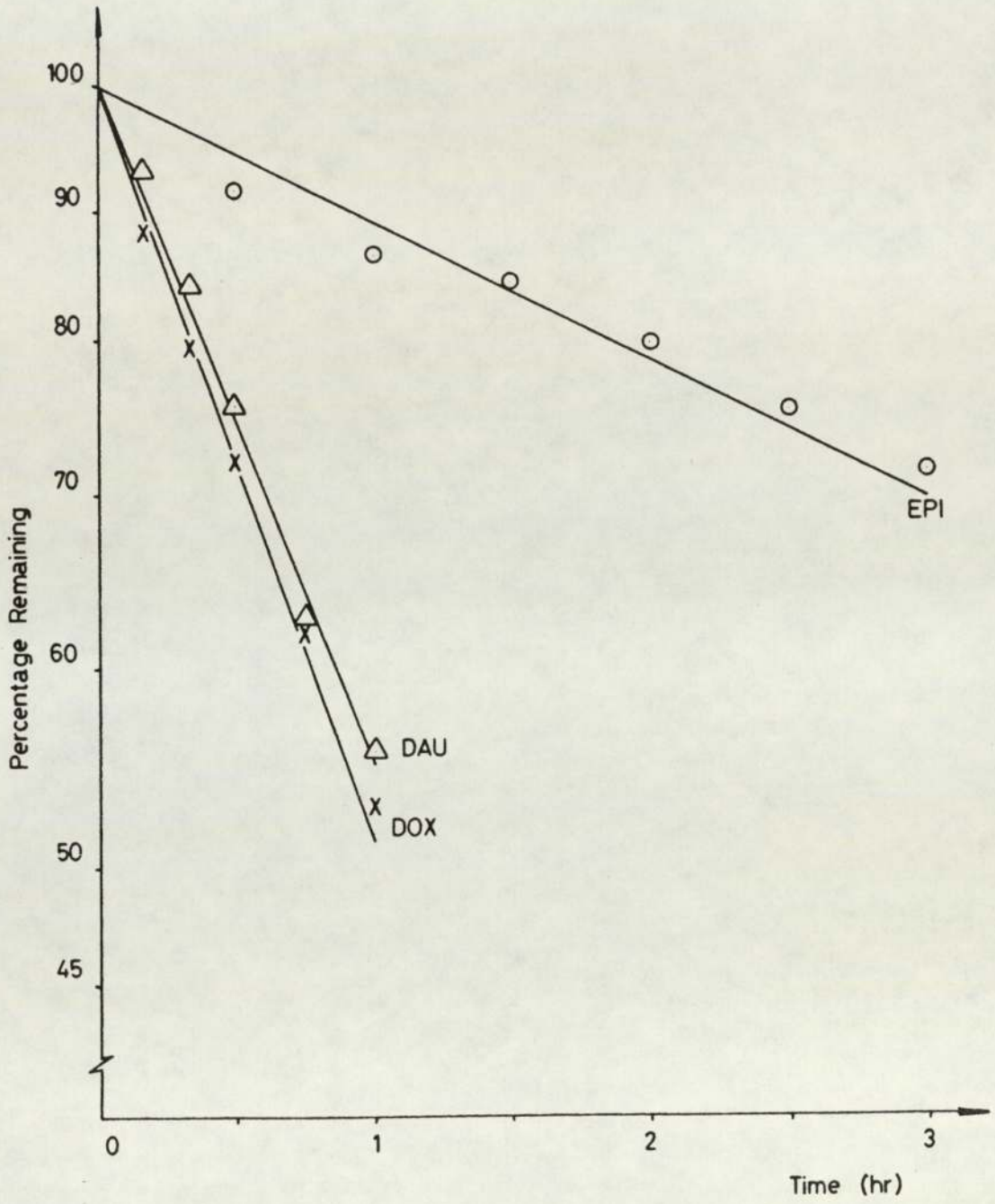


Figure 4.2. Natural logarithm of the percentage doxorubicin, daunorubicin and epirubicin remaining versus time in half strength Sorenson buffer pH 1.4 at 70°C.

Table 4.2 shows the rate constants for degradation of each drug in aqueous Sorenson buffer. The values for doxorubicin and epirubicin at pH 5.0 could not be determined as a gelatinous precipitate formed in these solutions (after 48 hours and 96 hours for doxorubicin and epirubicin respectively). This precipitate may have been due to poor solubility of either parent compound, aglycone degradation products, or both, in the buffer at this pH value. Table 4.3 shows the rate constants for degradation in identical Sorenson buffer solutions with 25% dimethylformamide (DMF) as co-solvent. Comparison of the data in Tables 4.2 and 4.3 show that the rate of degradation of all three anthracyclines was approximately one and a half times more rapid in aqueous buffer admixtures than in admixtures which contained the co-solvent.

These results become significant when the rate of degradation of these anthracyclines is studied in alkaline solution. Under these conditions, unless the initial drug concentration is less than or equal to 25μg/ml, it is necessary to include a co-solvent in the admixture, (section 4.3.ii), consequently, the rate of degradation is lower than that determined in equivalent aqueous buffer solutions. This fact must be borne in mind when the rate constants in the following section are considered.

The difference in the rate of degradation in water compared to the DMF:water mixture may be explained by the dielectric effect. The dielectric constant of dimethylformamide (DMF) is 26.6 at 25°C [187] and is much

lower than the dielectric constant of water which is 80 at 20°C [188]. If a change in the number of ions occurs during a reaction, such as that observed during the degradation of doxorubicin, daunorubicin and epirubicin the rate of reaction is affected by the dielectric constant of the solvent.

Ion formation during degradation proceeds more readily in a solvent with a higher dielectric constant (water in this case) than in the co-solvent water admixture as the ionic transition is more stabilized in water than in the co-solvent system. The stabilization of a charged transition state between two ions, A and B, may be expressed quantitively by the following equation;

$$\ln k = \ln k_{\varepsilon=\infty} - \frac{K Z_A Z_B}{\varepsilon}$$

where k is the measured degradation rate constant, $k_{\varepsilon=\infty}$ is the rate constant in a medium of infinite dielectric constant ε, Z_A and Z_B are the charges on the reacting species, K is a nominal constant holding Avogadro's number (N), electrical charge (e), the interionic distance within the activated complex (r), the gas constant (R) and temperature (T) such that $K = Ne^2 / rT$ [188].

pH of buffer	RATE CONSTANT k (hr^{-1})		
	DOX	DAU	EPI
1.2	8.17×10^{-4}	7.14×10^{-4}	5.99×10^{-4}
2.0	6.52×10^{-4}	3.77×10^{-4}	5.15×10^{-4}
3.0	6.03×10^{-4}	3.26×10^{-4}	2.98×10^{-4}
4.0	5.83×10^{-4}	2.08×10^{-4}	4.20×10^{-4}
5.0	--	5.99×10^{-4}	--

Table 4.2. Rate constants for degradation of doxorubicin, daunorubicin and epirubicin in half strength aqueous Sorenson buffer (pH 1.2 to 5.0) at 25°C.

pH of buffer	RATE CONSTANT k (hr^{-1})		
	DOX	DAU	EPI
1.2	5.32×10^{-4}	2.97×10^{-4}	3.12×10^{-4}
2.0	5.10×10^{-4}	3.10×10^{-4}	2.00×10^{-4}
3.0	3.81×10^{-4}	2.54×10^{-4}	2.02×10^{-4}
4.0	4.89×10^{-4}	1.72×10^{-4}	1.88×10^{-4}
5.0	7.05×10^{-4}	3.44×10^{-4}	6.38×10^{-4}

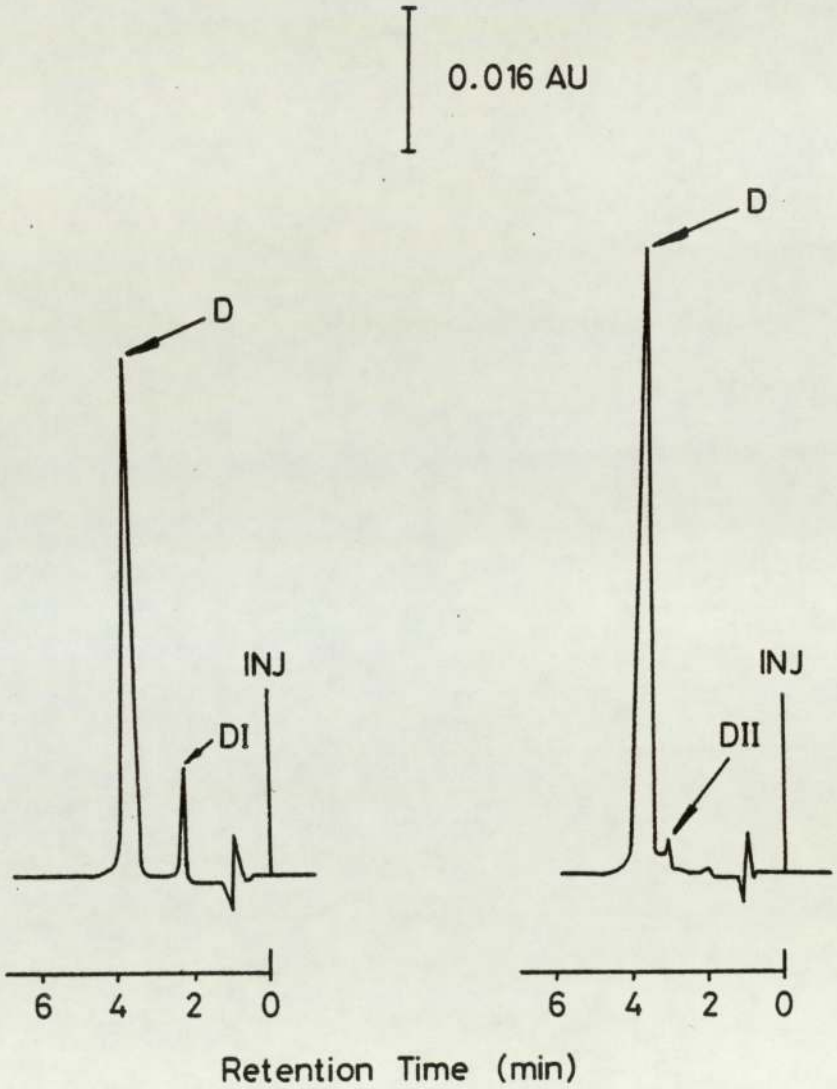
Table 4.3. Rate constants for degradation of doxorubicin, daunorubicin and epirubicin in half strength Sorenson buffer (pH 1.2 to 5.0), with 25% DMF, at 25°C.

Figure 4.3 shows chromatograms of decomposition mixtures of doxorubicin in aqueous Sorenson buffer pH 1.2 and pH 4.0. After 168 hours at pH 1.2 a degradation product (DI) was apparent which was well resolved from the parent compound. A similar pattern of degradation was also observed at pH 2.0 and 3.0. Chromatograms obtained from solutions at pH 4.0 showed a degradation product (DII) which eluted just before the parent compound. There was no evidence to suggest that DI was produced at this pH value. These data suggest that the mechanism of degradation of these anthracyclines at pH values less than 4 differs from the mechanism of degradation at pH values greater than 4. This observation has been recently reported by Beijnen et al [184] in studies on the degradation of daunorubicin and doxorubicin in aqueous solution and is discussed in greater detail later in this chapter.

The rate constants for degradation of doxorubicin, daunorubicin and epirubicin in Sorenson buffer pH 1.4 at 50, 60, 70 and 80°C are shown in Table 4.4. Figure 4.4 shows a typical first order plot obtained for daunorubicin under these conditions.

An Arrhenius plot was constructed from the rate constants obtained for the Sorenson buffer system at pH 1.4 to gain an insight into the order of magnitude of the thermodynamic processes that controlled the degradation of doxorubicin. A plot of the logarithm of the rate constant, k, versus the reciprocal of the absolute temperature (Figure 4.5) showed that the Arrhenius relationship was obeyed

between 50 and 80°C. From the slope and intercept of this plot the activation energy Ea and the frequency factor A were calculated as 99.56 kJ/mol and 2.512×10^{20} min^{-1} respectively.



a) pH 1.2 b) pH 4.0

Figure 4.3. High performance liquid chromatograms obtained for the degradation of doxorubicin in half strength Sorenson buffer at 25°C (100µg/ml at 168 hours). Mobile phase: acetonitrile:water 40:60 vol:vol; pH 2.5, flow rate 1.4ml/min., detection 290nm, chart speed: 5mm/min., sensitivity 0.16 AUFS, doxorubicin = D, degradation products = DI and DII.

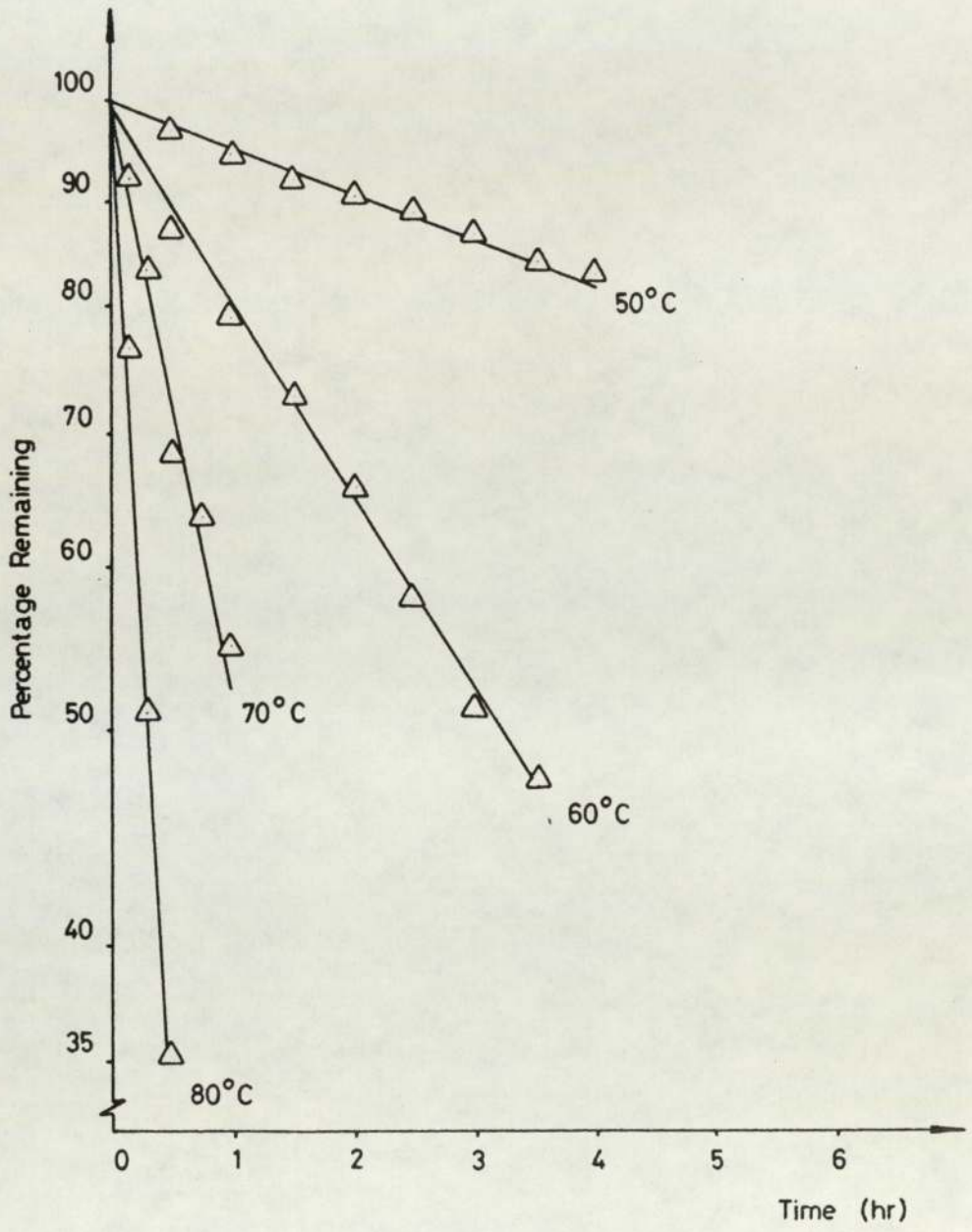


Figure 4.4. Natural logarithm of the percentage daunorubicin remaining versus time in half strength Sorenson buffer pH 1.4 at 50, 60, 70, and 80°C.

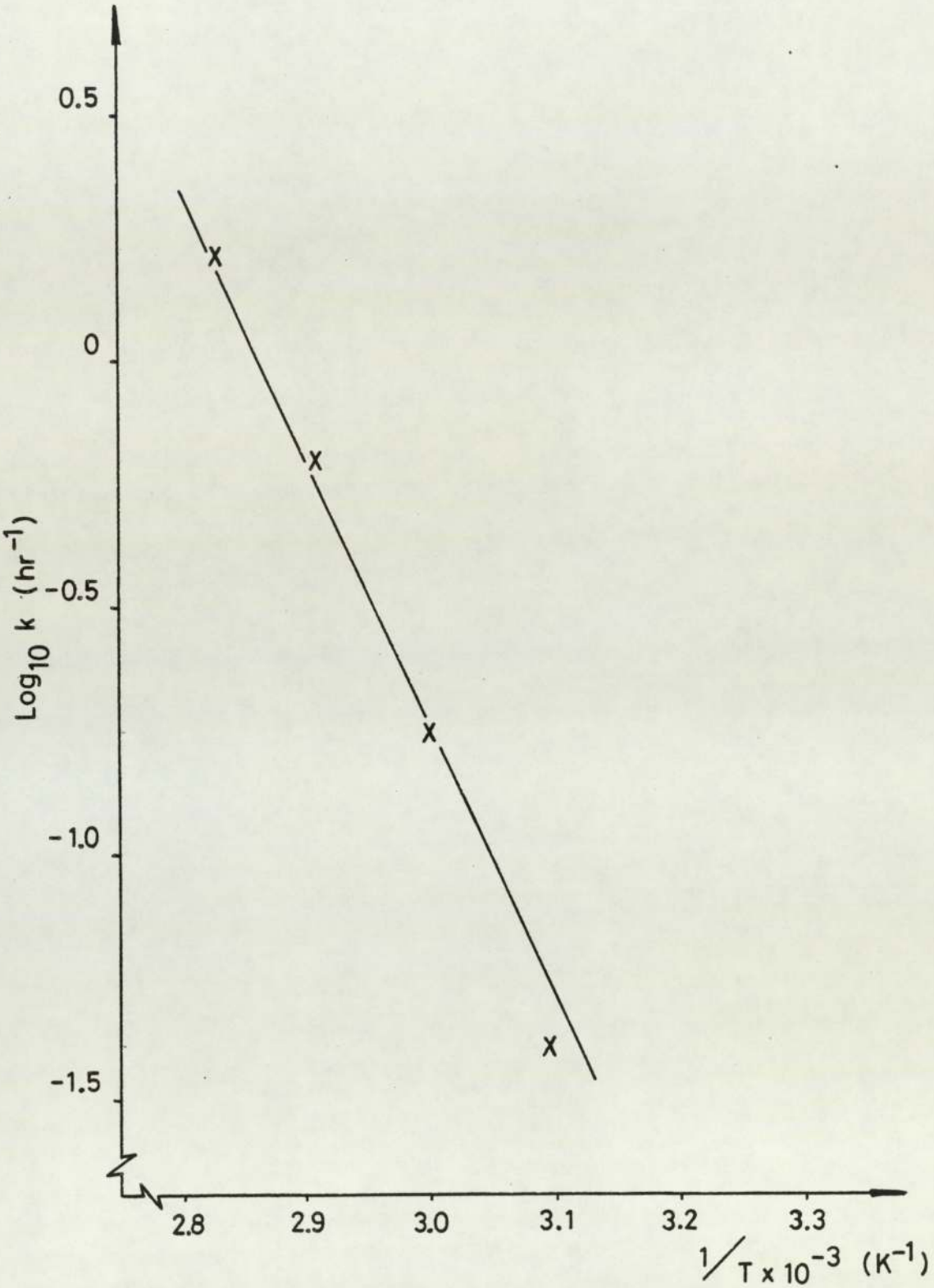


Figure 4.5. Arrhenius plot of the rate constants for degradation of doxorubicin (100µg/ml) in half strength Sorenson buffer at pH 1.4. T= absolute temperature (K).

Temp. °C	RATE CONSTANT k (hr^{-1})		
	DOX	DAU	EPI
50	4.06×10^{-2}	4.56×10^{-2}	1.68×10^{-2}
60	1.78×10^{-1}	2.10×10^{-1}	4.24×10^{-2}
70	6.38×10^{-1}	5.99×10^{-1}	1.09×10^{-1}
80	1.91	2.11	3.73×10^{-1}

Table 4.4. Rate constants for degradation of doxorubicin, daunorubicin and epirubicin in half strength Sorenson buffer pH 1.4 at 50, 60, 70, and 80°C.

Figure 4.6 shows a typical chromatogram of a decomposition mixture of daunorubicin after one hour at pH 1.4 and 60°C. Similar traces were obtained for doxorubicin and epirubicin. A review of the literature revealed a similar pattern of degradation for daunorubicin at pH values less than 4 [184] and suggested that the degradation product (DrI) was the aglycone daunorubicinone. Under the same conditions doxorubicin produced doxorubicinone (DI) and epirubicin produced epirubicinone (EI) (which had the same structure as doxorubicinone). The mechanism of degradation of doxorubicin in acidic solution is shown in Figure 4.7.

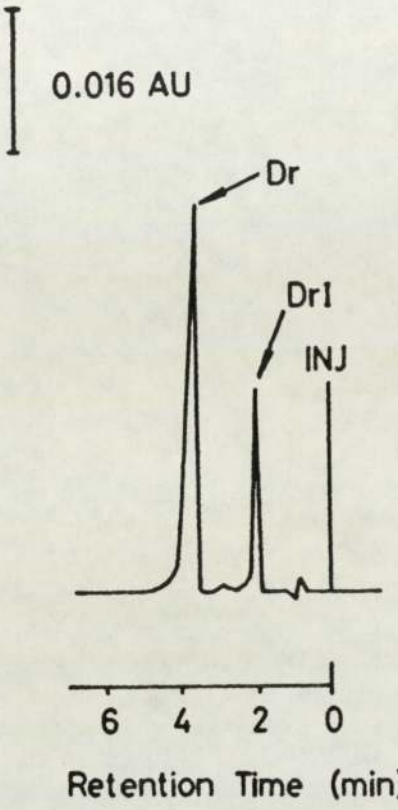


Figure 4.6. High performance liquid chromatogram of a decomposition mixture of daunorubicin after one hour in Sorenson buffer pH 1.4 at 60° C. Mobile phase: acetonitrile:water 55:45 (vol:vol; pH 2.5), flow rate 1.5ml/min, chart speed 5mm/min., detection 290nm, sensitivity 0.16 AUFS, daunorubicin= Dr, degradation product= DrI.

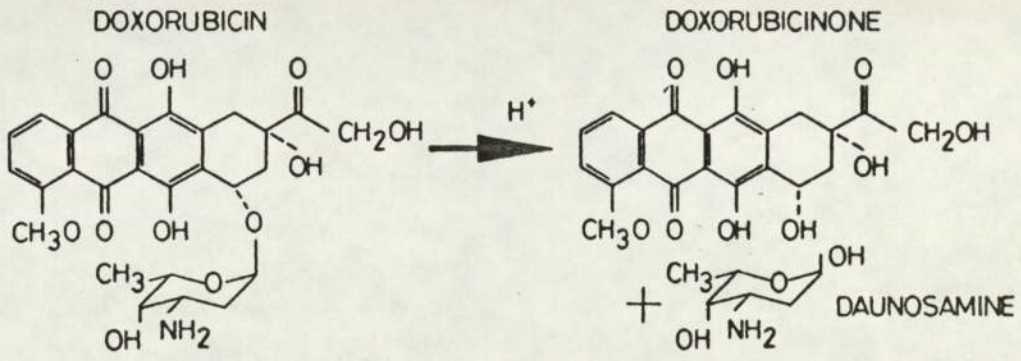


Figure 4.7. Degradation of doxorubicin in acidic solution.

Beijnen et al [189] also observed that if daunorubicin was subjected to more protracted and vigorous acid conditions (100°C for 28 hours in 1M HCl) daunorubicinone was subjected to progressive degradation. The structures of the resultant compounds (DrI, daunorubicinone, DrII, 7-epidaunorubicinone, DrIII, 7,8,9-bis-anhydrodaunorubicinone, DrIV, 7-deoxydaunorubicinone and, DrV, (structure not completely certain) are shown in Figure 4.8 (below).

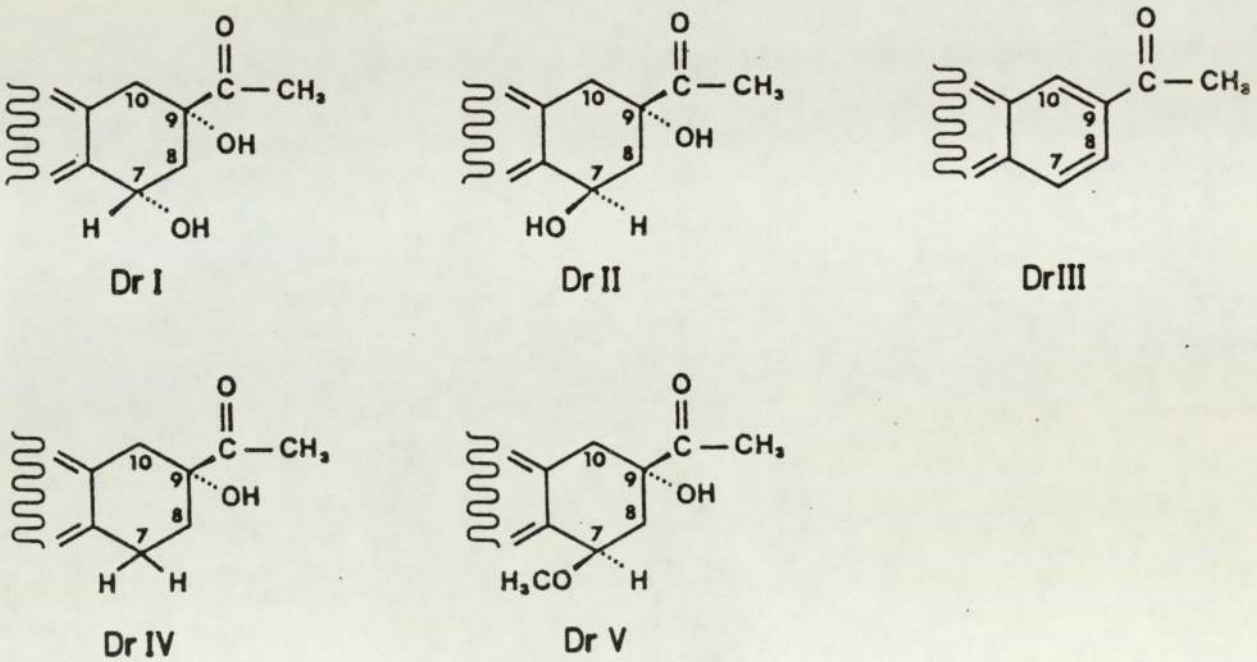


Figure 4.8. Structures of degradation products of daunorubicin in acidic solution (from Beijnen et al [189]).

4.3.ii. Degradation in alkaline media.

Plots of the natural logarithm of the percentage parent drug remaining versus time were linear (r=0.999) which indicated that degradation in alkaline solution followed first order kinetics. Figure 4.9 shows a typical first order plot for doxorubicin in Tris buffer with 25% DMF at 25°C.

Table 4.5 shows the rate constants for degradation of each drug in aqueous Britton-Robinson buffer. The rate of degradation of doxorubicin and epirubicin at pH 6.37, 7.96 and 9.91 and daunorubicin at pH 7.96 and 9.91 could not be accurately determined due to the gradual formation of a floccular precipitate. Precipitation of anthracyclines in alkaline solution below pH 10 has been documented [185] but prevention of this problem has not been investigated.

Results from systematic studies which investigated the use of co-solvents to prevent precipitation in alkaline solution showed that the appearance of a precipitate was dependent on the pH of the buffer, the drug and buffer concentrations. Beijnen et al [189] made similar observations in a study of the degradation of doxorubicin in phosphate buffer pH 8.0. Precipitates were observed, by these authors, in solutions containing 50, 100 and 500μg/ml of doxorubicin but not in solutions containing between 1μg/ml and 20μg/ml.

It has also been observed that doxorubicin forms dimers [190] and possibly larger stacked aggregates [191] in

concentrated solutions. Porumb [191] postulated that this aggregation was driven by hydrophobic forces and opposed by the electrostatic repulsion between the positively charged doxorubicin molecules. It has also been suggested that the 14'-hydroxyl of doxorubicin is either directly or indirectly involved in dimerisation [190]. The dimerisation constant for the monomeric form of doxorubicin in equilibrium with its dimeric form has also been shown to be dependent on the buffer composition and its ionic strength [190].

These data suggest that in solutions of doxorubicin, 50μg/ml, in Britton-Robinson buffer pH 7.96 or pH 9.91, (ionic strength 0.5M) in this experiment, dimerisation was favoured by a combination of the high ionic strength and the drug concentration. Subsequently, aggregate formation occurred which resulted in the gradual appearance of a precipitate as time elapsed.

Beijnen et al [185] also observed that the appearance of these precipitates, which contained substantial amounts of both parent compound and degradation products, was also related to the type of container used for the study. These authors observed that the degradation products present in the precipitate were very hydrophobic and strongly adsorbed onto silanised glass showing a dull pink coating. In non-silanised glass vials precipitation occurred. This observation accounts for the formation of a floccular precipitate in Britton-Robinson buffer at pH 6.37, 7.96 and 9.91 which was observed in this study.

The rate of degradation of all three anthracyclines was readily determined in Britton-Robinson buffer at pH 11.98 as

precipitation did not occur at this pH value. A review of the literature revealed that at pH values greater than 10 Beijnen et al [185] also observed that degradation products were neither adsorbed nor precipitated in glass containers. These authors postulated that this was due to the ionisation of a phenolic function of the degradation products which enhanced their solubility.

The occurrence of precipitates in alkaline solution, whatever their constituents, precluded accurate determination of degradation kinetics. For this reason methanol and dimethylformamide (DMF) were investigated as potential co-solvents to prevent precipitation in Britton-Robinson buffer. Methanol (10 to 50%) was found to be unsuitable as a co-solvent as it failed to prevent precipitation. Although DMF prevented precipitation transparent needle-like crystals, which were probably due to phosphates from buffer salts, formed in all solutions. For this reason Tris buffer was substituted to assess the rate of degradation of these drugs in alkaline solution. Results from systematic studies with DMF revealed that 25% of this co-solvent was the optimum concentration to prevent precipitation. At higher concentrations the admixtures did not freeze and results from chromatographic analysis of these solutions suggested that degradation continued to occur during storage.

Table 4.6 shows the rate constants for degradation of all three drugs in Tris buffer with 25% DMF at pH 7.2, 8.0 and 9.0. As this experiment proceeded the admixtures showed

a progressive colour change from orange through orange-red to purple. The extent of this colour change appeared to correlate with the amount of decomposition measured by HPLC. It has been suggested that the rapid colour change which occurs immediately on addition of these anthracyclines to strongly alkaline solution is indicative of rapid destruction of the chromophore [189]. However, results from a subsiduary experiment showed that a solution which was alkalised then immediately acidified regained its orange colour without evidence of degradation on HPLC or changes in the UV spectrum. This suggested that the anthracyclines possess indicator-like properties.

Figure 4.10 shows the protolytic equilibria of doxorubicin in aqueous solution. The spectrophotometric investigations of Sturgeon and Schulman [192] indicated that the only species present in significant amounts in aqueous solution at pH values less than 7.0 is the singly charged species (1) with the positive charge at the amino group. At higher pH values this monocation can either lose a proton from the amino group to form the neutral species (2) or from a phenolic group to form a zwitterion (3). Either of these species may then lose a proton to form the singly charged anion (4). The negative charge in (3) and (4) can be placed at either the C-11 or at the C-6 phenolic oxygen. As doxorubicin, daunorubicin and epirubicin contain several ionisable functions, overlap of ionic equilibria is possible. In doxorubicin the amino group and a phenolic group are both ionised in the region pH 8.0 to 13.0 but because the amino group of the sugar moiety is several atoms

removed from the aromatic chromophore of doxorubicin, inductive influences, due to the free or protonated amino group upon the electronic spectrum of doxorubicin are negligible. Thus, only the dissociation of the phenolic group affects the spectrophotometric properties of the molecule in alkaline solutions and therefore the rapid colour change from orange-red to purple observed at pH values greater than 10 is due to ionisation of one of the phenolic functions in the anthraquinone ring system. The second phenolic function in this system has been observed to ionise at pH values greater than 13 [12]. Ionisation of the second phenolic group was not observed in this experiment but adjustment of solutions of doxorubicin to pH values greater than 12.5 with sodium hydroxide solution resulted in a further colour change from deep-purple to deep blue.

Figures 4.11 and 4.12 show first order plots for the degradation of each drug in Tris buffer with 25% DMF at pH 8.0 and aqueous Britton-Robinson buffer at pH 11.98 respectively. Both these plots show clearly that the rates of degradation of doxorubicin and epirubicin in alkaline solution were similar whereas the rate of degradation of daunorubicin was substantially slower. The only structural difference between doxorubicin and daunorubicin is the presence of a hydroxyl group on C14 in the doxorubicin molecule. This structural modification therefore must hold the key to the differences in stability which were observed for doxorubicin, epirubicin and daunorubicin in alkaline solution in this experiment.

The increased stability of daunorubicin in alkaline solution may be explained as follows; As indicated later in this section Beijnen et al [184] observed that the ketol side-chain present in doxorubicin favoured enolisation while in daunorubicin this tautomerisation is not likely to occur. Enolisation of the C-9 side-chain in doxorubicin leads to the formation of the enolate anion (Figure 4.16) which promotes the formation of 7,8-dehydro-9,10-desacetyl-daunorubicinone, rather than the bis-anhydro derivatives which are also formed during the degradation of daunorubicin in alkaline solution. These data, therefore, appear to suggest that the degradation of daunorubicin and doxorubicin in alkaline solution follow different mechanisms. As a hydroxyl group is also present on the C-14 carbon atom in epirubicin this analogue is expected to undergo enolisation and to follow the same mechanism of degradation as doxorubicin in alkaline solution.

As stated above enolisation leads to production of the enolate anion. As only doxorubicin and epirubicin undergo enolisation, and the rate of degradation of these analogues in alkaline solution was much more rapid than daunorubicin, this suggests that this anion formation may also lead to an increase in the rate of oxidation of doxorubicin and epirubicin compared to daunorubicin (section 3.3).

In summary, enolisation of the C-9 side-chain of doxorubicin and epirubicin appeared to affect both the rate and mechanism of degradation of these analogues in alkaline solution.

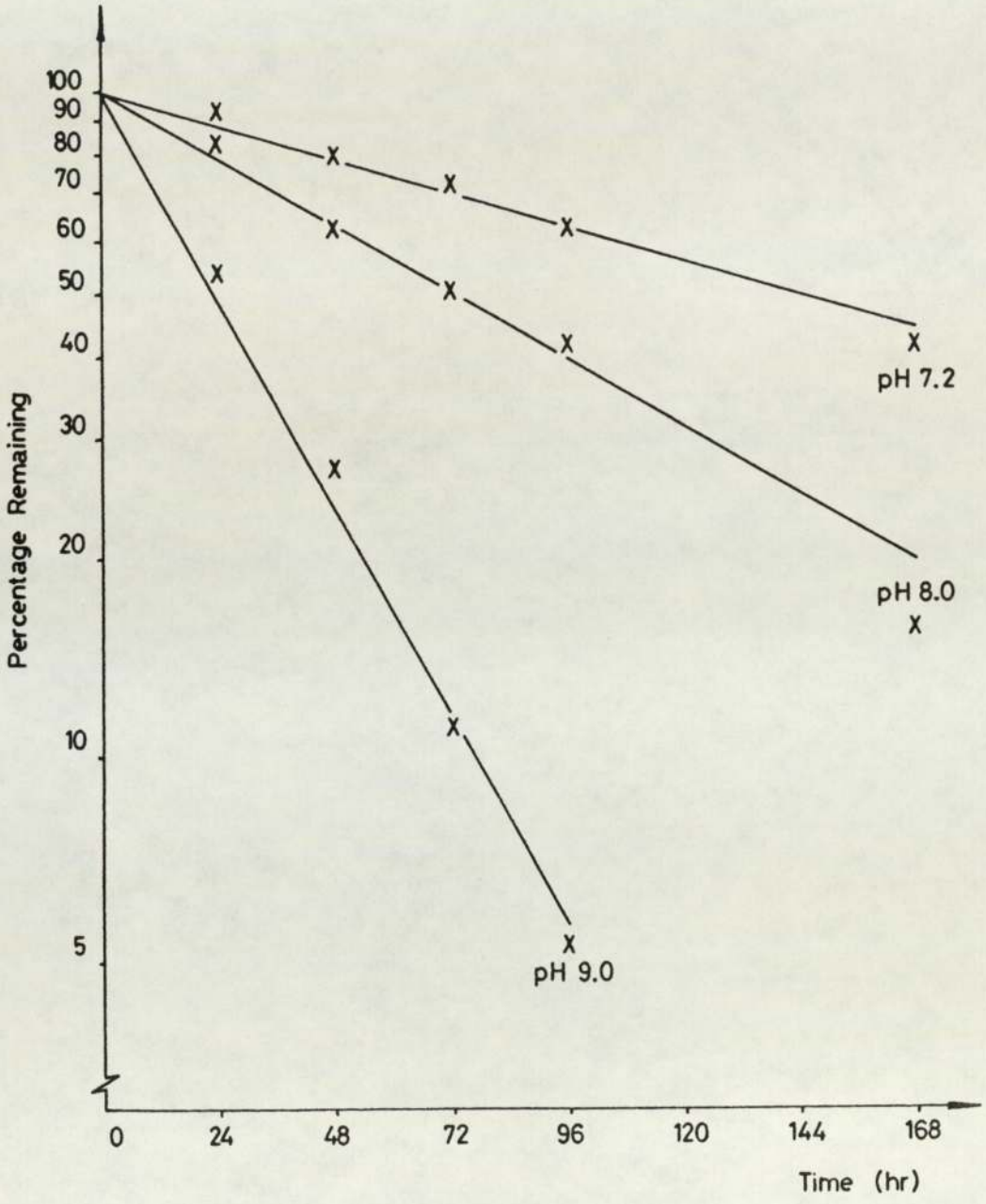


Figure 4.9. Natural logarithm of the percentage doxorubicin remaining versus time in half strength Tris buffer (pH 7.2, 8.0 and 9.0), with 25% DMF, at 25°C.

pH of buffer	RATE CONSTANT k (hr^{-1})		
	DOX	DAU	EPI
6.37	--	5.08×10^{-4}	--
7.96	--	--	--
9.91	--	--	--
11.98	9.58×10^{-1}	1.33×10^{-1}	1.50

Table 4.5. Rate constants for degradation of doxorubicin, daunorubicin and epirubicin in half strength aqueous Britton-Robinson buffer (pH 6.37 to 11.98) at 25°C.

Note: -- rate of degradation not calculated due to precipitation.

pH	RATE CONSTANT k (hr^{-1})		
	DOX	DAU	EPI
7.2	5.35×10^{-3}	3.57×10^{-4}	4.80×10^{-3}
8.0	10.91×10^{-3}	7.43×10^{-4}	9.87×10^{-3}
9.0	31.21×10^{-3}	9.27×10^{-4}	20.16×10^{-3}

Table 4.6. Rate constants for degradation of doxorubicin, daunorubicin and epirubicin in half strength Tris buffer (pH 7.2, 8.0 and 9.0), with 25% DMF, at 25°C.

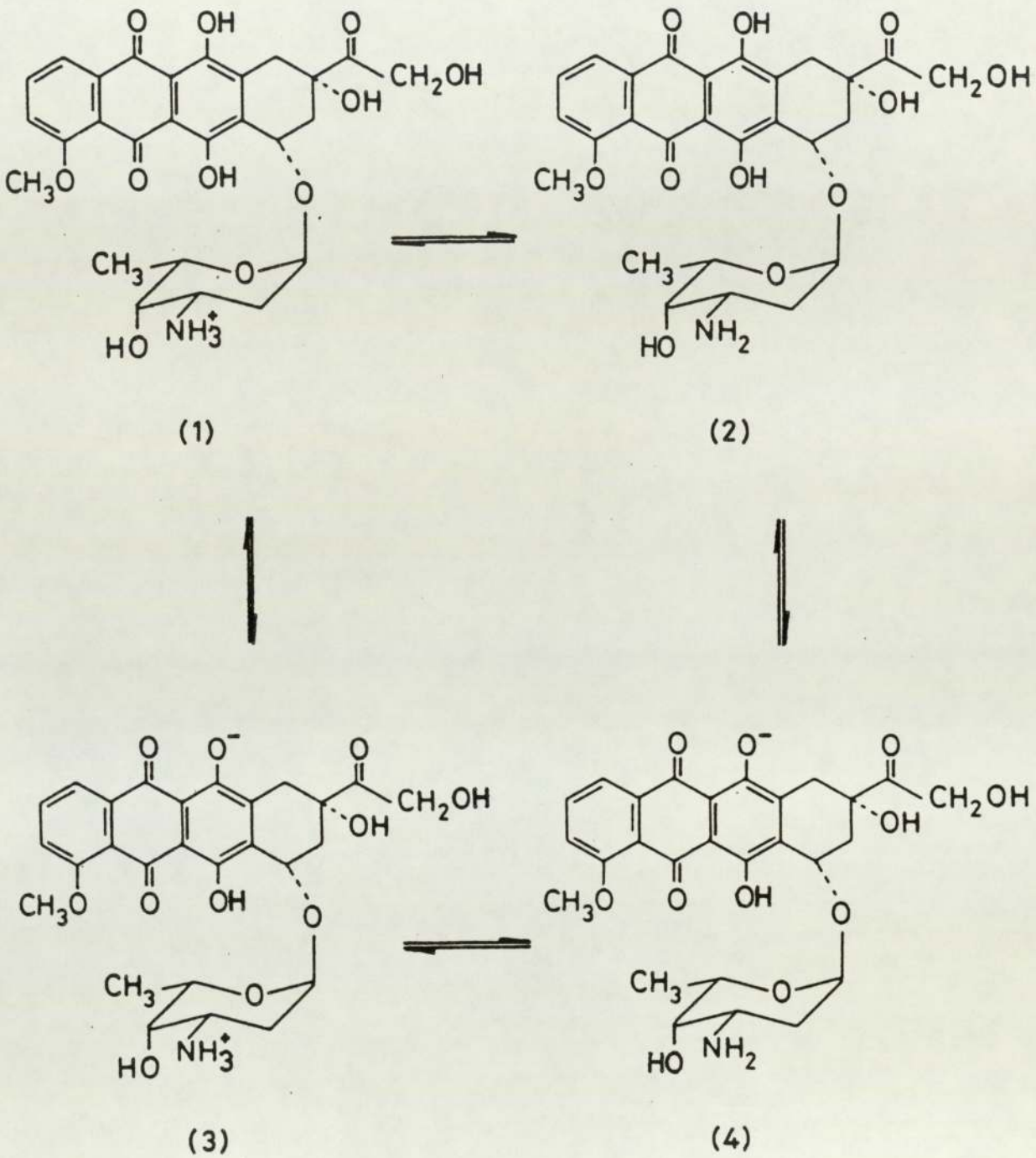


Figure 4.10. Protolytic equilibria of doxorubicin in aqueous solutions (from Arcamone [12]).

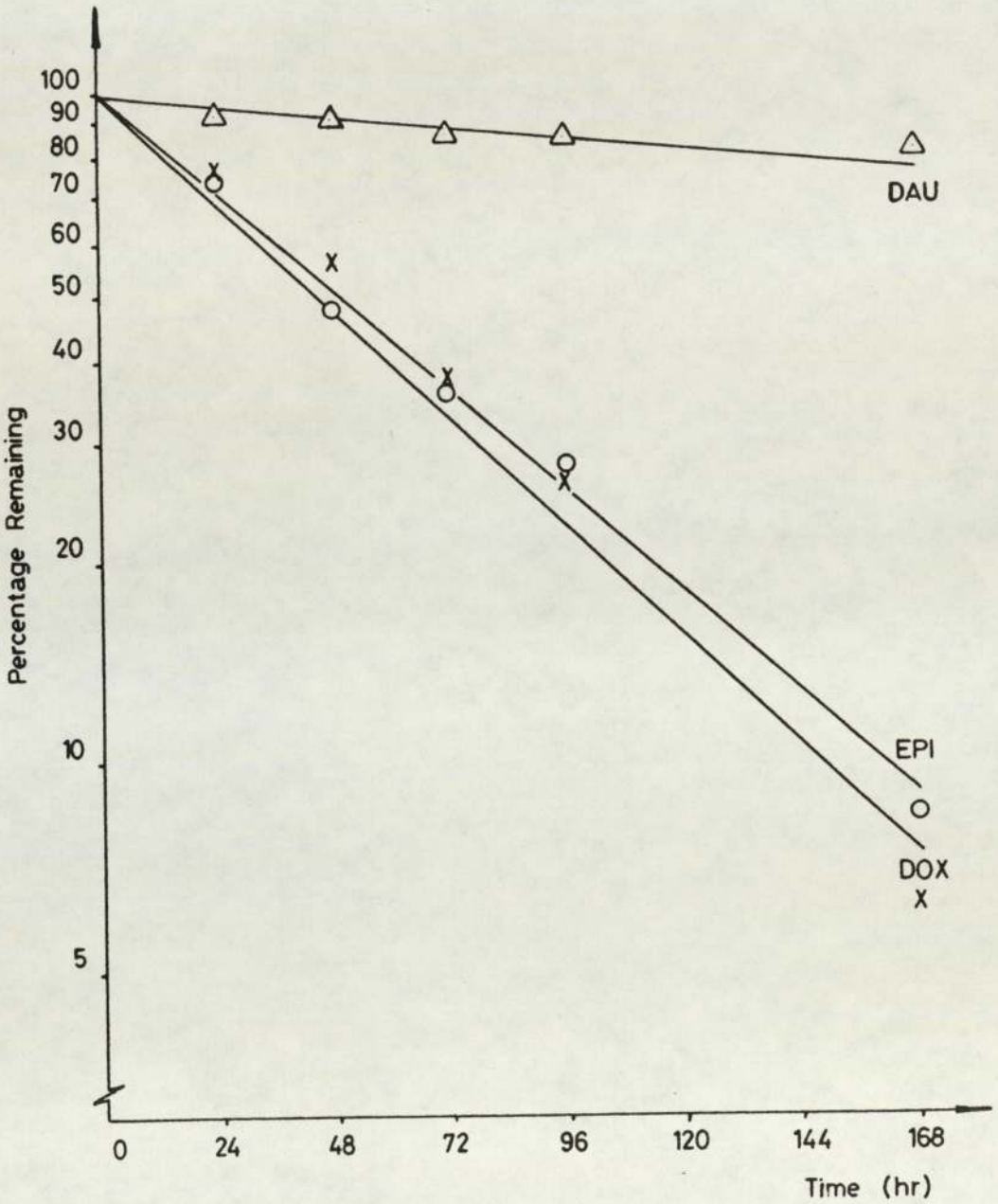


Figure 4.11. Natural logarithm of the percentage doxorubicin, daunorubicin and epirubicin remaining versus time in half strength Tris buffer pH 8.0, with 25% DMF, at 25°C.

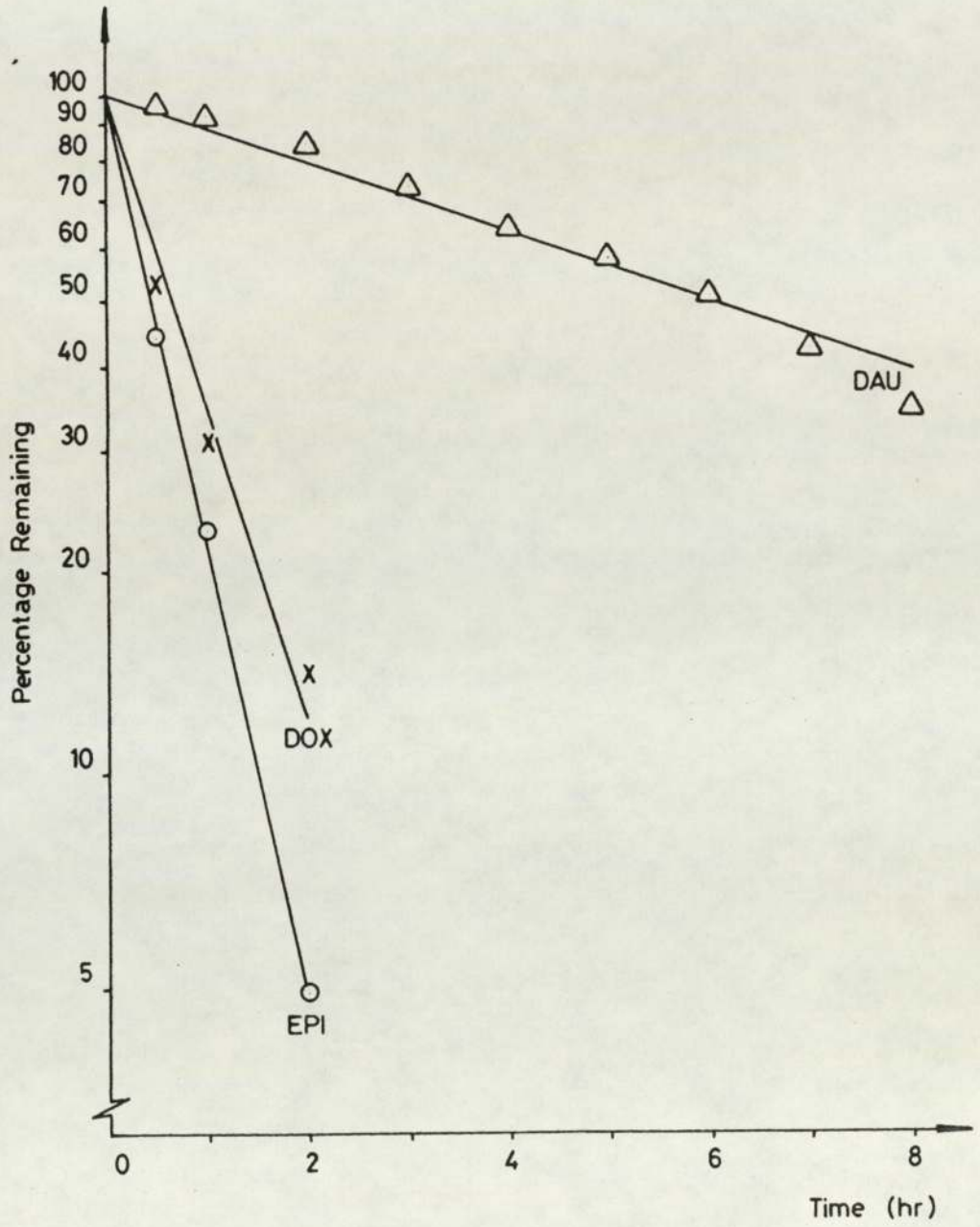


Figure 4.12. Natural logarithm of the percentage doxorubicin, daunorubicin and epirubicin remaining versus time in half strength Britton-Robinson buffer, pH 11.98, at 25°C.

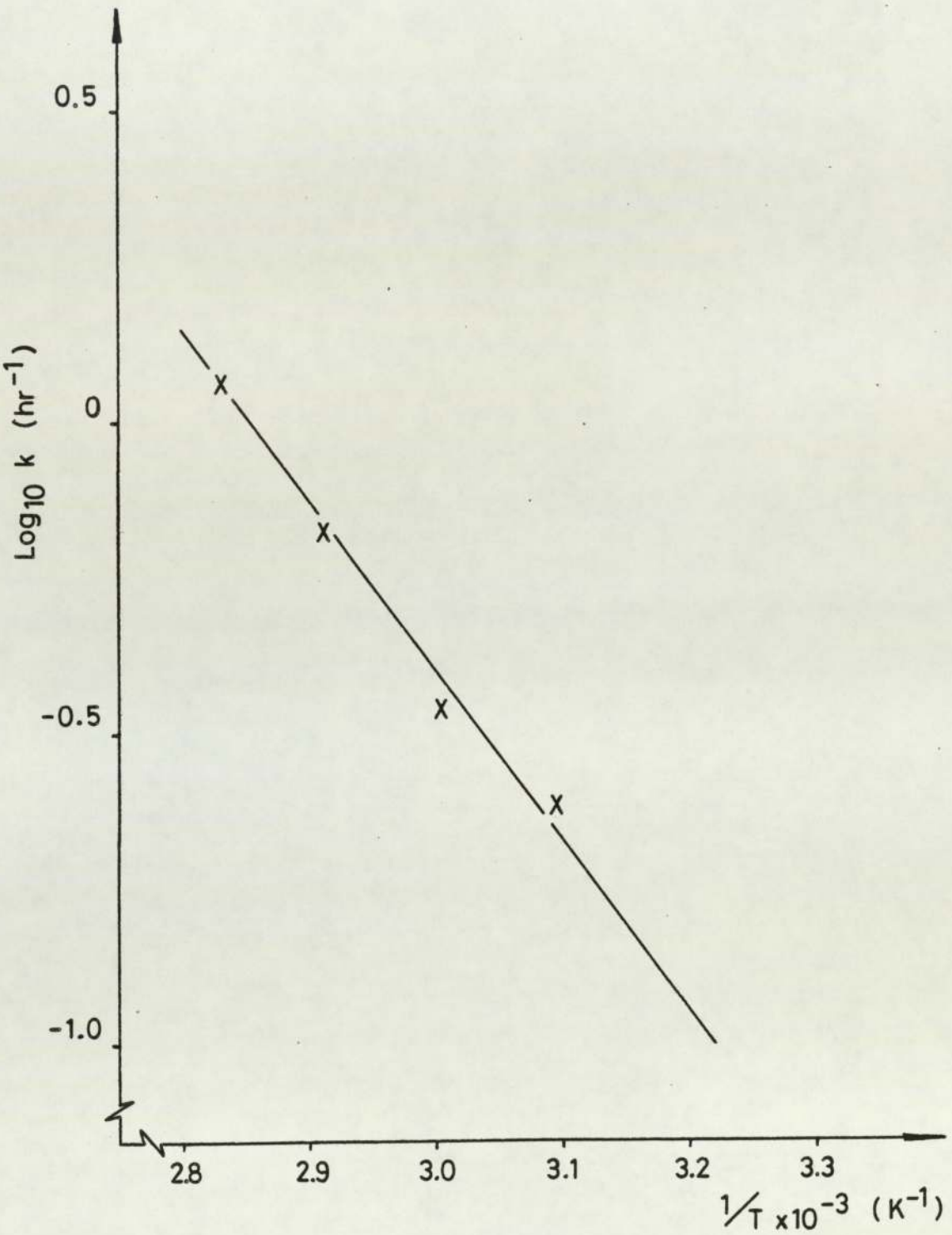


Figure 4.13. Arrhenius plot of the rate constants for degradation of doxorubicin (100µg/ml) in half strength Tris buffer, pH 8.5, T = absolute temperature (K).

The rate constants for degradation of doxorubicin, daunorubicin and epirubicin at 50, 60, 70 and 80° C in Tris buffer, pH 8.5, are shown in Table 4.7. An Arrhenius plot was constructed from the rate constants obtained for this system. Figure 4.13 shows that the Arrhenius relationship was obeyed between 50 and 80°C. From the slope and intercept of this plot the activation energy (Ea) and the frequency factor (A) were calculated as 55.76 kJ/mol and 2.511×10^{12} min^{-1} respectively. Gradual formation of a precipitate was observed in all solutions as the experiment progressed. Although sampling was stopped as soon as precipitation was observed the data suggest that the rate constants for degradation were inaccurate. Consequently the activation energy calculated from a graphical plot of the logarithm of these values versus the reciprocal of absolute temperature was lower than expected.

TEMP. (°C)	RATE CONSTANT k (hr^{-1})
50	2.41×10^{-1}
60	3.27×10^{-1}
70	6.69×10^{-1}
80	1.15

Table 4.7. Rate constants for degradation of doxorubicin, daunorubicin and epirubicin in half strength Tris buffer, pH 8.5, at 50, 60, 70 and 80°C.

Beijnen et al [189] recently identified the degradation products of daunorubicin at 50µg/ml in 0.01M phosphate buffer pH 8.0. After four hours at 100°C precipitates formed which redissolved after extraction into chloroform. TLC analysis of the extract showed seven zones. The major degradation products identified by these authors (DrVI, DrVII and DrVIII) are shown in Figure 4.14. DrVI was isolated and demonstrated chromatographic and spectrographic properties which were identical to DrIII and the reference compound 7,8,9-bisanhydrodaunorubicinone. DrVII appeared to be identical to DrIV, 7-deoxydaunorubicinone. DrVIII was identified as 7,8-dehydro-9,10-desacetyldaunorubicinone. Low yields of compounds in the other zones noted on TLC prevented full characterisation and structure elucidation.

The degradation pattern of doxorubicin at pH 8.0 has not been elucidated completely. Decomposition mixtures extracted with chloroform and analysed by TLC showed one pink-coloured major degradation product DVIII, 7,8-dehydro-9-10-desacetyldaunorubicinone (Figure 4.14) and minor quantities of other fluorescent compounds. This compound was also the major degradation product found in degradation mixtures of epirubicin. The bis-anhydro derivative of doxorubicin DVI (Figure 4.15) was not found as a degradation product in alkaline solution. This fact was explained by Beijnen et al [184] as follows: The ketol side chain present in doxorubicin and epirubicin favours enolisation while in daunorubicin this tautomerisation is not likely to occur. Enolisation of the C9 side chain in doxorubicin

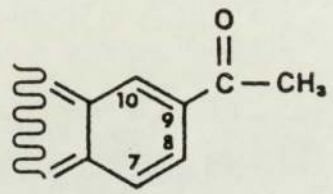
promotes the formation of 7,8,-dehydro-9-10-desacetyldaunorubicinone, rather than the bis-anhydro derivative in alkaline solution. The mechanism for the keto-enol tautomersiation of doxorubicin is shown in Figure 4.16.

If this were the case the bis-anhydro derivative of epirubicin would not be expected to form in alkali degraded solutions of this drug as both doxorubicin and epirubicin possess identical aglycones. To investigate this hypothesis chromatograms of each drug were examined. If formed, 7,8,-dehydro-9,10-desacetyldaunorubicinone and the respective bis-anhydro derivatives of each drug would elute after the parent compound. For this reason, peaks which eluted before the parent compound are not discussed.

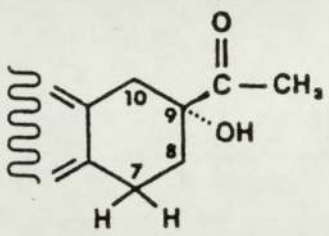
The decomposition mixture of daunorubicin at pH 9.0 (Figure 4.17) was expected to show both DrVIII and DrVI. The absence of these peaks in Figure 4.17 was probably due to the fact that the pH of the Tris buffer used in this experiment was two units lower than in the literature cited and consequently very little degradation had occurred. It may be possible to obtain more detailed data for daunorubicin by studying degradation in buffer solutions at higher pH values or at increased temperature although degradation in strongly alkaline media was difficult to control.

Chromatograms of doxorubicin (Figure 4.18) showed, as expected, only one broad peak, DVIII (probably 7,8-dehydro-9,10-desacetyldaunorubicinone) which eluted after the parent compound. Chromatograms obtained for a decomposition mixture of epirubicin, showed two broad peaks which eluted after the

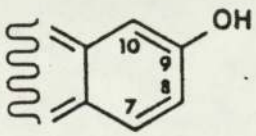
parent compound. The first peak (EVIII) may be 7,8-dehydro-9,10-desacetyldaunorubicinone and the second may be the bis-anhydro derivative of epirubicin (EVI). If this is the case the enolisation theory of Beijnen et al [184] does not appear to hold for this analogue, but, without proof of structure, it is impossible to make any firm conclusion about the identity of these compounds.



Dr VI

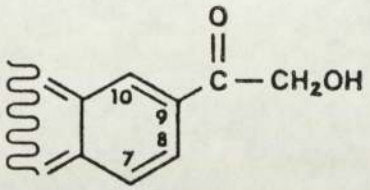


Dr VII



Dr VIII

Figure 4.14. Structure of the degradation products of daunorubicin in alkaline solution (from Beijnen et al [189]).



D VI

Figure 4.15. The structure of 7,8,9- bisanhydro-doxorubicinone.

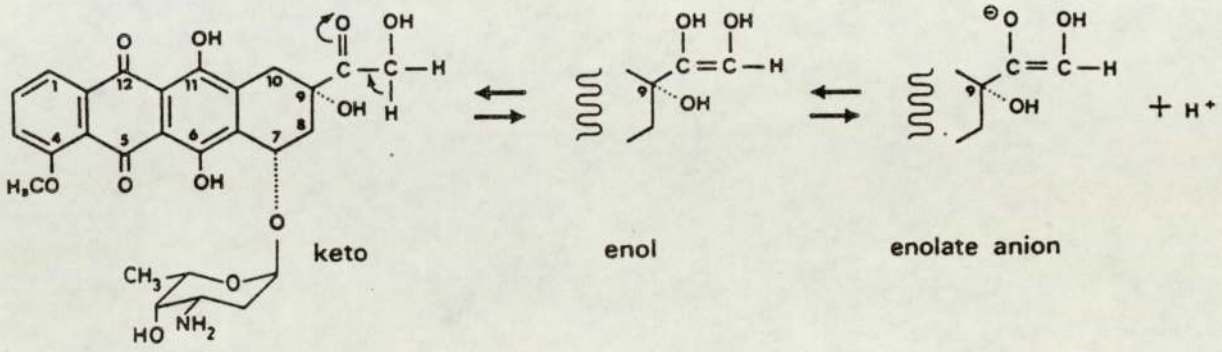
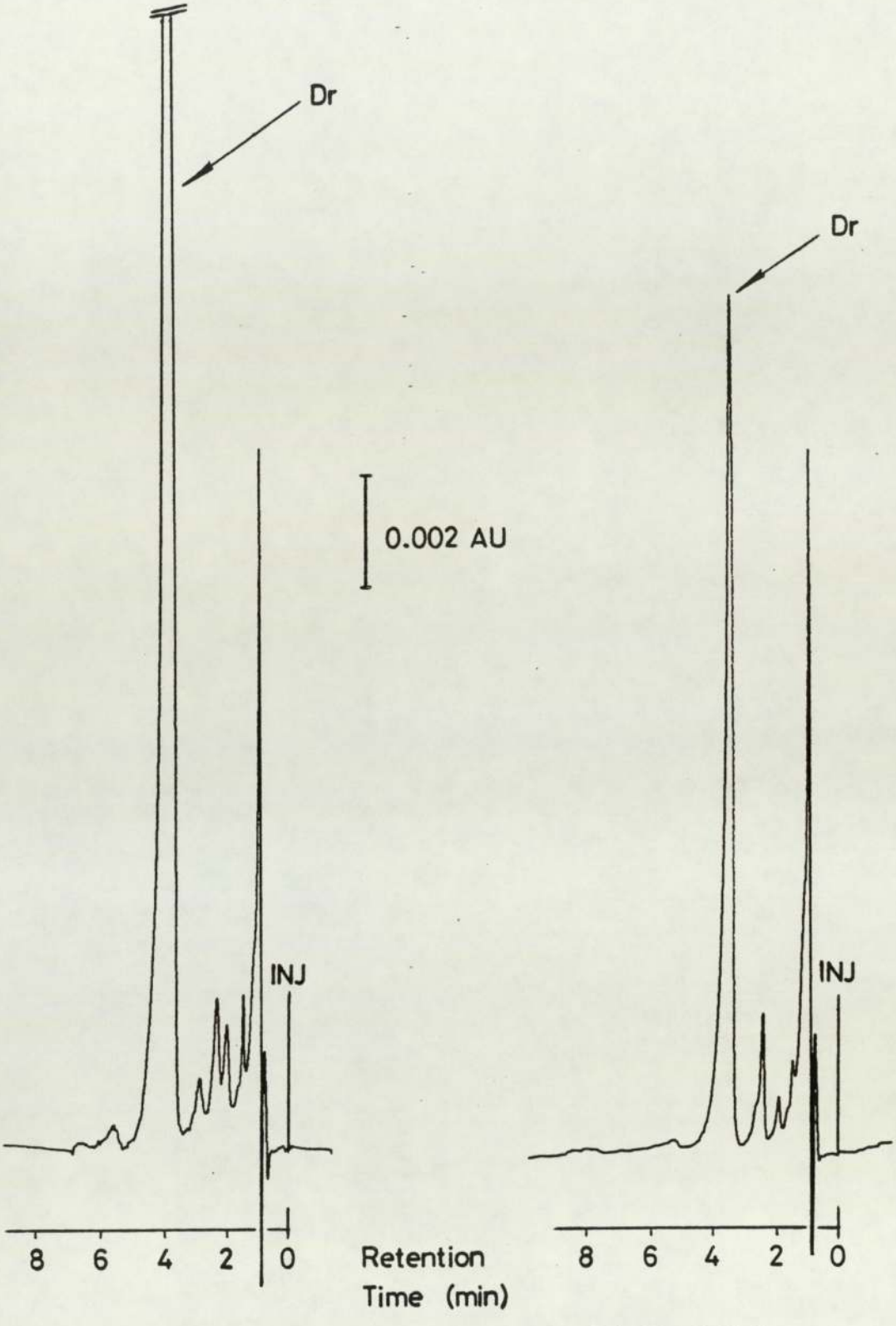


Figure 4.16. Keto-enol tautomerization and ionisation of doxorubicin (from Beijnen et al [184]).



a) T= 168 hours

b) T= 336 hours

Figure 4.17. High performance liquid chromatograms obtained for the degradation of daunorubicin in half strength Tris buffer, with 25% DMF, pH 9.0 at 25 ° C. (100µg/ml at 168 hours). Mobile phase: acetonitrile:water 55:45 vol:vol; pH 2.5, flow rate: 1.5ml/min., detection: 290nm, chart speed: 5mm/min., sensitivity: 0.02 AUFS, daunorubicin = Dr.

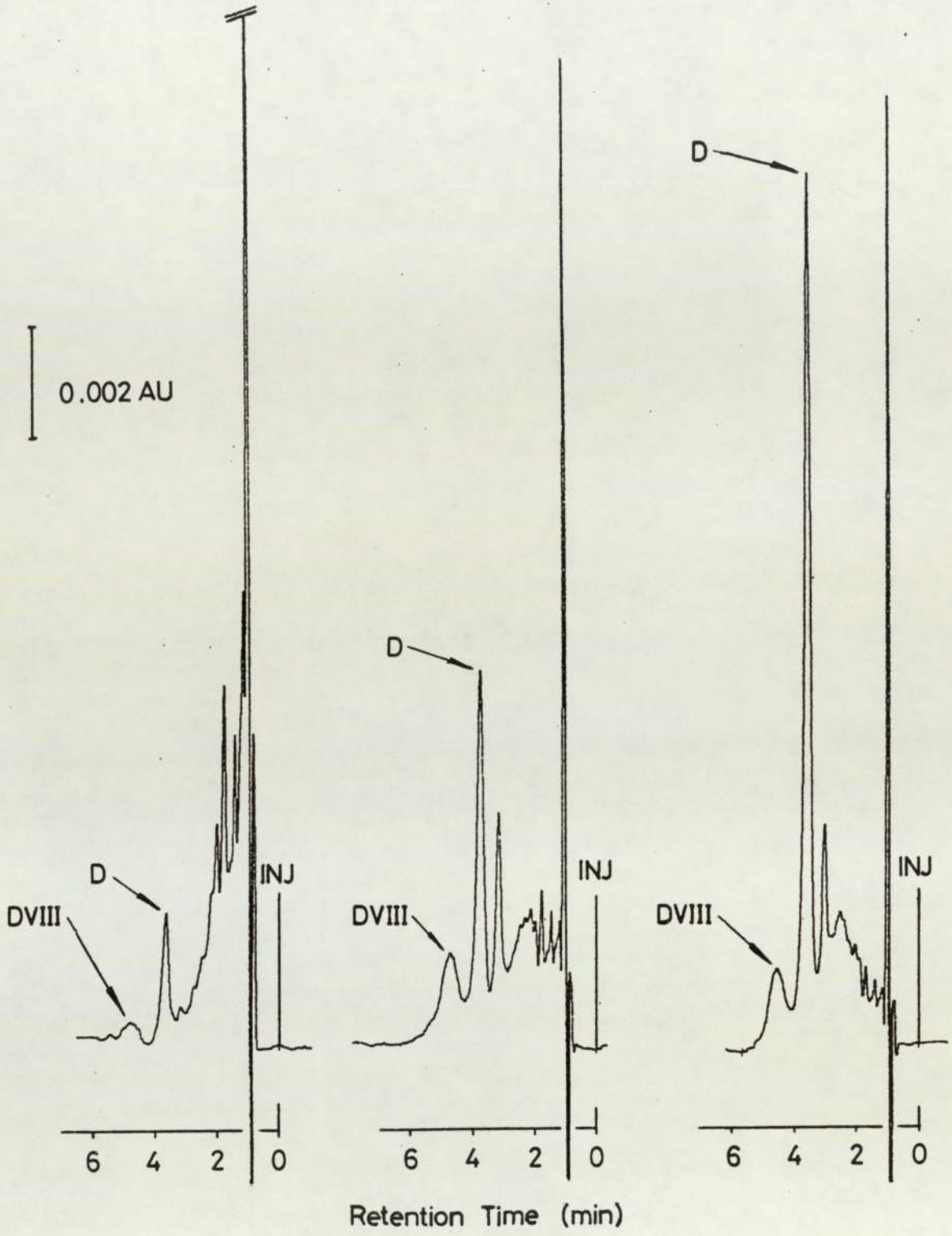


Figure 4.18. High performance liquid chromatograms for doxorubicin in half strength Tris buffer with 25% DMF at pH 7.2, 8.0 and 9.0 at 25° C (100µg/ml at 168 hours). Mobile phase: acetonitrile:water 40:60 vol:vol; pH 2.5, flow rate: 1.4ml/min., detection: 290nm, chart speed: 5mm/min., sensitivity: 0.02 AUFS, doxorubicin = D, degradation products = DVIII.

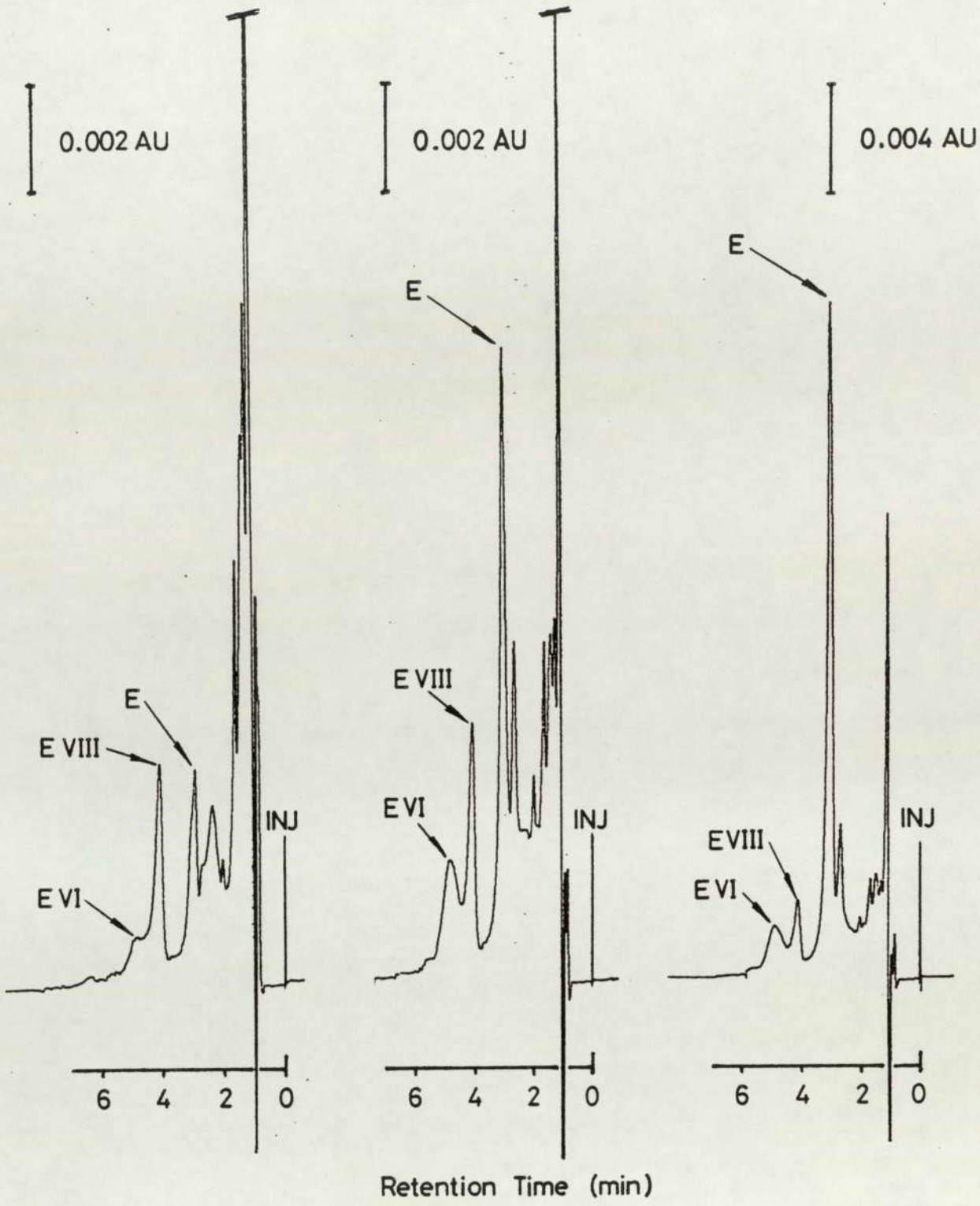


Figure 4.19. High performance liquid chromatograms for epirubicin in half strength Tris buffer with 25% DMF at pH 7.2, 8.0 and 9.0 at 25° C, (100µg/ml at 168 hours). Mobile phase: acetonitrile:water 50:50 vol:vol; pH 2.5, flow rate: 1.3ml/min., detection: 290nm; chart speed: 5mm/min., epirubicin = E, degradation products = EVIII and EIV.

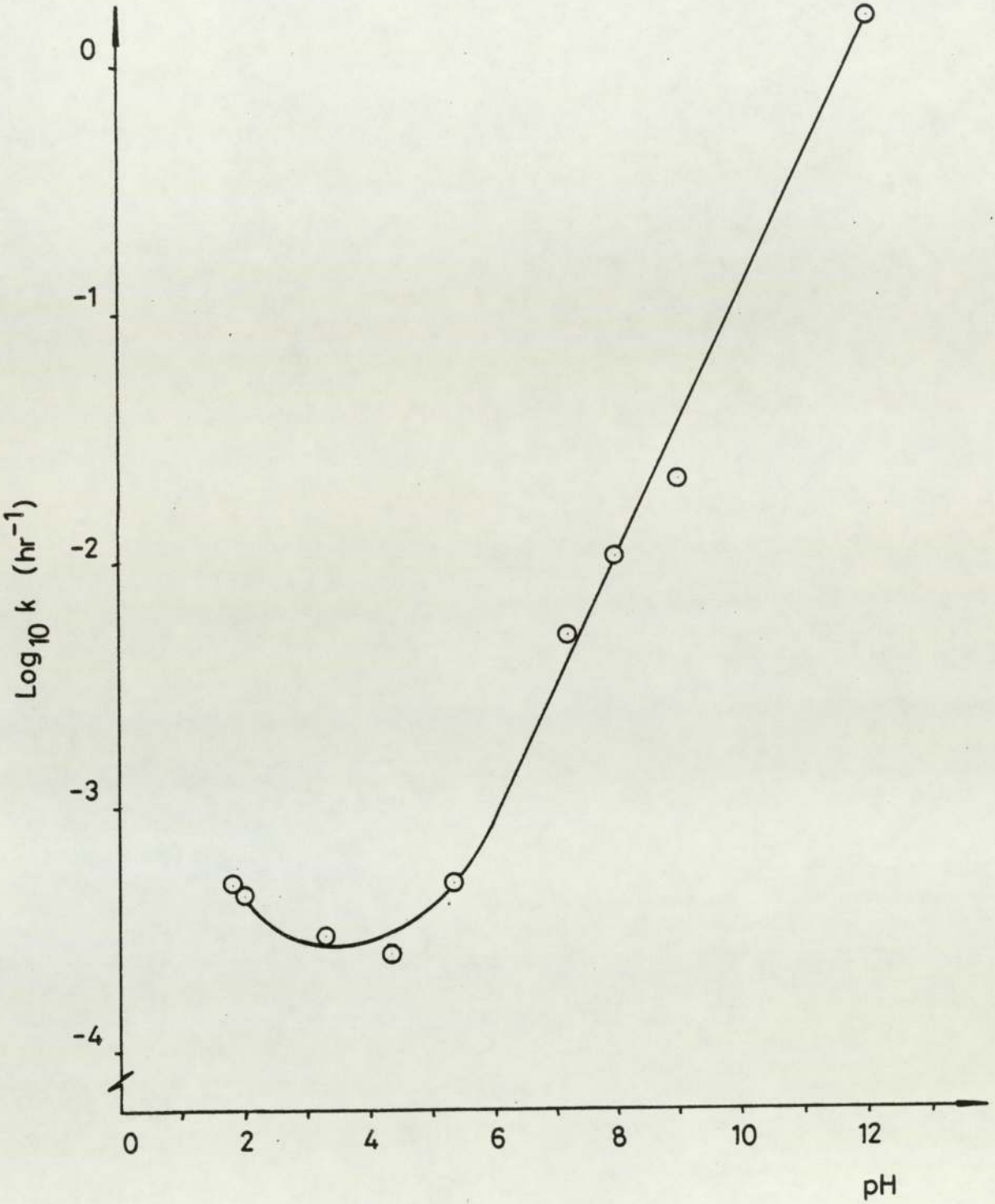


Figure 4.20. Logarithm of the rate constant for degradation, k, versus the pH of the buffer for epirubicin.

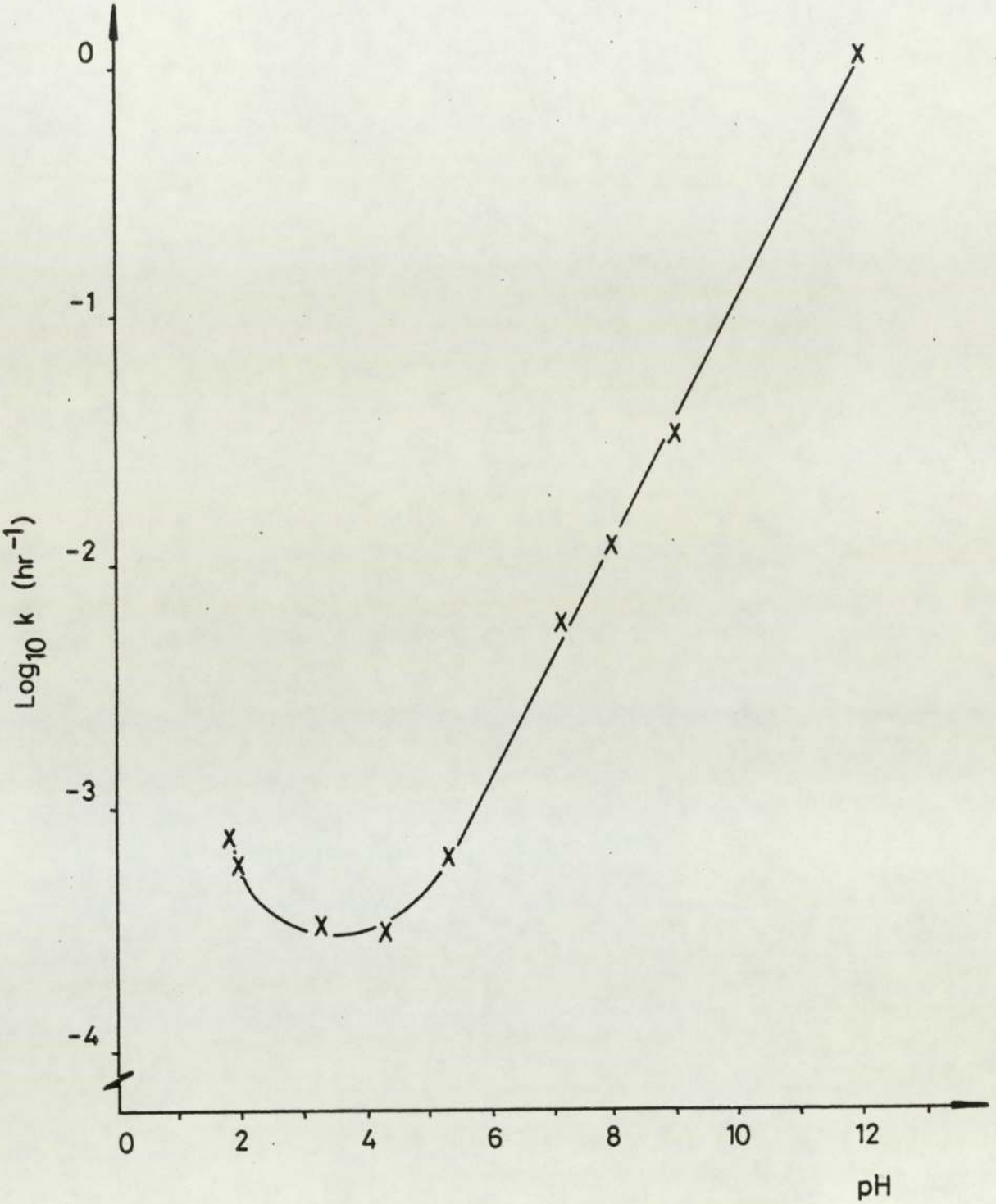


Figure 4.21. Logarithm of the rate constant for degradation, k, versus the pH of the buffer for doxorubicin.

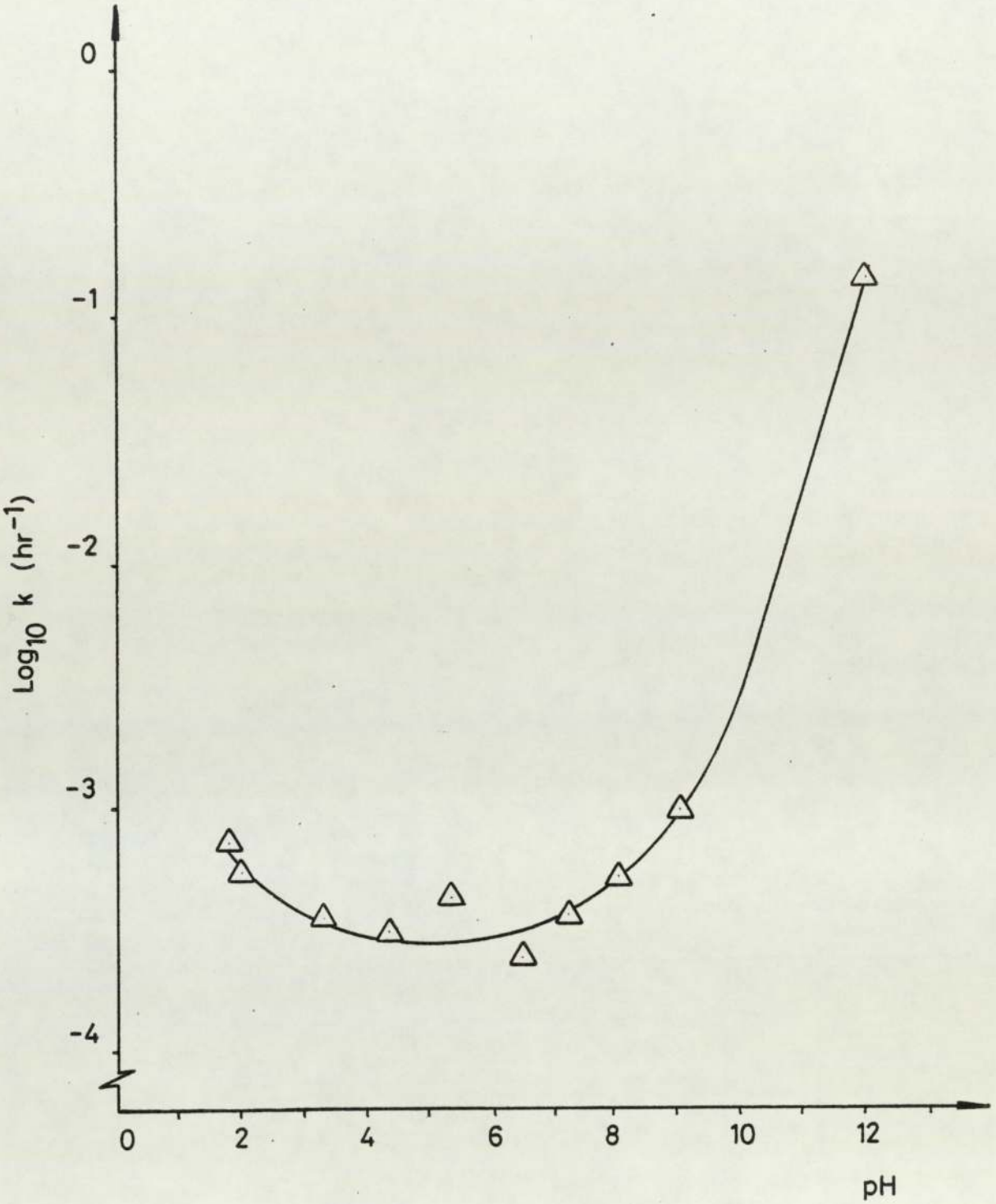


Figure 4.22. Logarithm of the rate constant for degradation, k, versus the pH of the buffer for daunorubicin.

The pH profiles (Figures 4.20, 4.21, and 4.22) show that the rate of degradation of doxorubicin, daunorubicin and epirubicin is strongly influenced by the pH of the medium. All three drugs appeared to be optimally stable at pH values between 4.0 and 5.0.

Doxorubicin and daunorubicin degraded at almost the same rate in acidic solution whereas the rate of degradation of epirubicin was substantially slower. This suggested that the rate of cleavage of the glycosidic bond in acidic media was strongly dependent on structural modifications in the amino sugar moiety and affected little by structural modifications in the aglycone portion of the molecule.

X-ray diffraction studies by Neidle and Taylor [193] and Courseille et al [194] revealed the importance of hydrogen bonding as a determinant of both molecular conformation and crystal structure of the anthracyclines. Although these authors determined the crystal structure under different conditions the fundamental topological parameters did not change substantially indicating the high stability of the observed conformation which in turn suggested that this conformation was also likely to be favoured in solution. These studies showed that the cyclohexene ring (ring A) was in the half chair conformation and the sugar moiety appeared to be nearly perpendicular to the plane of the chromophore. An increased stability of the half chair conformation of ring A was considered to be given by an O-9, O-7 hydrogen bond. Alternatively, the C9 hydroxyl may display an intramolecular hydrogen bond with the ring

oxygen atom of the amino sugar moiety. This second type of intramolecular linkage would contribute to stabilise a given orientation of the amino sugar moiety with respect to the aglycone residue. Another important feature is represented by the hydrogen bond exhibited by the positively charged amino group and the C4' hydroxyl. In the epirubicin molecule the C4' hydroxyl has an equatorial orientation whereas in doxorubicin the C4' hydroxyl is axial. The two are otherwise identical. Therefore it appears that this structural difference may lead to a difference in the hydrogen bonding between this hydroxyl group and the amino group in the sugar moiety which may in turn lead to a difference in distribution of electronic charges in the quinonic chromophore. The net result of these changes in the charge distribution appeared to result in stabilisation of the glycosidic bond in the epirubicin molecule.

In alkaline solution doxorubicin and epirubicin degraded at almost the same rate whereas the rate of degradation of daunorubicin was substantially slower. This suggested that structural differences in the aminosugar moiety did not affect the rate of degradation in alkaline solution. The only structural difference between doxorubicin and daunorubicin is the presence of a hydroxyl group on C-14 in the doxorubicin molecule. As a result of this structural difference enolisation of the C-9 side-chain of doxorubicin, and presumably epirubicin, has been postulated to occur [184]. Enolisation promotes formation of 7,8-dehydro-9,10-desacetyldaunorubicinone, rather than the bis-anhydro derivatives which are also formed during the degradation of

daunorubicin in alkaline solution. These data suggest that the degradation of daunorubicin and doxorubicin in alkaline solution follow different mechanisms. As epirubicin also contains a hydroxyl on the C-14 carbon atom this analogue is expected to undergo enolisation and therefore to follow the same mechanism of degradation as doxorubicin.

As only doxorubicin and epirubicin undergo enolisation, and the rate of degradation of these two analogues in alkaline solution was much more rapid than daunorubicin, this suggests that anion formation during enolisation may also lead to an increase in the rate of oxidation of doxorubicin and epirubicin compared to daunorubicin as discussed in section 3.3.

In summary, enolisation of the C-9 side-chain of doxorubicin and epirubicin appeared to effect both the rate and mechanism of degradation of these analogues in alkaline solution.

Chapter Five

Stability of doxorubicin, daunorubicin and epirubicin in infusion fluids.

5.1. Introduction.

Results of recent studies which compared the efficacy and toxicity of prolonged infusions and intravenous bolus therapy of doxorubicin, indicated that infusion therapy reduced the incidence of cardiotoxicity without a reduction in clinical efficacy [26,43,78,125,130,133-135] (section 1.6.v). The purpose of the present study was to support further research in this field by studying the long term stability of doxorubicin and related compounds in infusion fluids. The effect of storage temperature, drug concentration, pH and type of solvent on the stability of each drug was investigated. In addition, the effects of repeated freezing and thawing on stability were studied.

5.2. Experimental.

Vials of each drug were reconstituted with Water For Injections (section 2.2.i.) to produce stock solutions which were used immediately to prepare the admixtures for testing. Either 5ml of a 2mg/ml solution of doxorubicin or epirubicin, or 2ml of a 5mg/ml solution of daunorubicin, were added to 100ml polyvinylchloride (PVC) minibags to yield initial theoretical drug concentrations of 95.2μg/ml, 95.2μg/ml and 98μg/ml respectively. The admixture solutions were prepared with the following intravenous fluids: Dextrose 5% injection BP (pH 4.36), sodium chloride 0.9% BP

(pH 5.2 and 6.47) (both Viaflex, Travenol) or Water For Injections (WFI) (Antigen). Admixtures were stored at either ambient temperature (normally 25°C), in the refrigerator at 4°C, or in the freezer at -20°C.

The effect of repeated freezing and thawing at ambient temperature was investigated in 100ml PVC minibags at a concentration of approximately 100µg/ml as follows: The percentage of each drug remaining after two freeze-thaw cycles (at 336 and 672 hours), which was used as the control, was compared to the percentage remaining in identical solutions which were frozen and thawed eleven times over a period of six weeks.

Solutions which were allowed to thaw at ambient temperature took about four hours to thaw completely. In order to speed up the thawing process the minibags were immersed (excluding the additive port) in a water bath at 25° C. In this way solutions could be completely thawed in approximately half an hour. Care was taken to ensure that the solutions were not overheated by removal of the minibags from the water before thawing was complete and allowing final re-equilibration at ambient temperature.

In a pilot study drug loss due to adsorption of doxorubicin onto PVC was investigated, under accelerated conditions, by comparison of the initial rate of drug loss in glass containers (approximately 100µg/ml) with an excess of coarsely chopped PVC (8.0g) with drug loss in identical control solutions. These studies were expanded to include a study of adsorption onto the surface of a minibag by comparison of the initial rate of drug loss in solutions of

doxorubicin 50, 100 or 200μg/ml in sodium chloride 0.9%, pH 5.2, at 4°C.

A large initial drug loss was observed for solutions of doxorubicin (approximately 100μg/ml) which were stored in polypropylene syringes. As adsorption onto polypropylene has not been reported adsorption onto the rubber on the barrel of the syringe was suspected. This hypothesis was investigated in an accelerated study which compared the initial rate of drug loss in solutions of doxorubicin in glass containers (approximately 100μg/ml) with an excess of coarsely chopped rubber (7.3g), from a rubber plunger of a 60ml polypropylene syringe, with drug loss in identical control solutions.

The stability of each drug was also investigated, in Water For Injections in disposable syringes, at a concentration of 2mg/ml. Immediately after reconstitution, the contents of two vials of either doxorubicin or epirubicin (both containing 50mg in 25ml) were drawn up to into 60ml polypropylene syringes (Becton-Dickinson Plastipak). Reconstituted vials of daunorubicin each contained 20mg of drug in 4ml therefore it was necessary to draw up the contents of five vials into the syringe and make up to volume (50ml) with Water For Injections to achieve a final concentration of 2mg/ml. After all air was excluded the syringes were sealed with Luer-lok hubs and stored at 4°C. The stablity of epirubicin, 2mg/ml, in Water For Injections in the manufacturer's container was also investigated at 4°C.

The colour and pH of each solution was noted immediately after mixing and periodically during the course of the experiment. All samples were protected from light with aluminium foil throughout the study and during thawing prior to assay.

Samples were removed for chromatographic analysis according to the following schedule: 2ml aliquots of each drug were collected initially, at 24 hour intervals for 192 hours, then weekly thereafter for six weeks. All samples were immediately frozen, stored at -20°C, and thawed at ambient temperature immediately prior to assay by HPLC. Samples were assayed in two batches in order to maximise the efficiency of chromatographic runs and reduce any inter-assay variability (section 2.3.ii). Experimental runs for each drug fluid admixture were duplicated.

Chromatograms were inspected for evidence of degradation products. Concentrations remaining were determined by interpolation of calculated peak areas on calibration curves which were constructed daily. All concentrations, which were expressed as a percentage of the initial concentration at time zero, represented the mean of duplicate values. Significant degradation was defined as a loss of equal to or greater than 10% of the original concentration. Where possible, the period of time for 10% disappearance of the original concentration of the drug (t90% value) was determined graphically from plots of the percentage parent drug remaining versus time.

Data were evaluated using a first order kinetic model by construction of plots of the natural logarithm of the

percentage parent drug remaining versus time. The rate constant, k, for drug loss was calculated by linear regression analysis of the first order plot.

Significant differences in the doxorubicin, daunorubicin and epirubicin content of the analysed samples were calculated by means of a one-way, two-way and/or a three-way analysis of variance (with replicates). Where it was necessary only to test the difference between two means for significance Student's t test was employed. In this way the results obtained in this experiment were compared with the data obtained by other authors. The pooled standard deviation for the two test samples and the standard error of the difference between the two sample means were calculated according to the usual equations [181].

5.3. Results and discussion.

5.3.i. Stability in polyvinylchloride (PVC) minibags.

Plots of the natural logarithm of the percentage parent drug remaining versus time, in PVC minibags (100µg/ml), exhibited a biphasic degradation pattern. Figure 5.1 shows the typical appearance of data plotted as a first order model for doxorubicin in sodium chloride 0.9%, pH 6.47, at 4° C. Similar plots were obtained for daunorubicin and epirubicin.

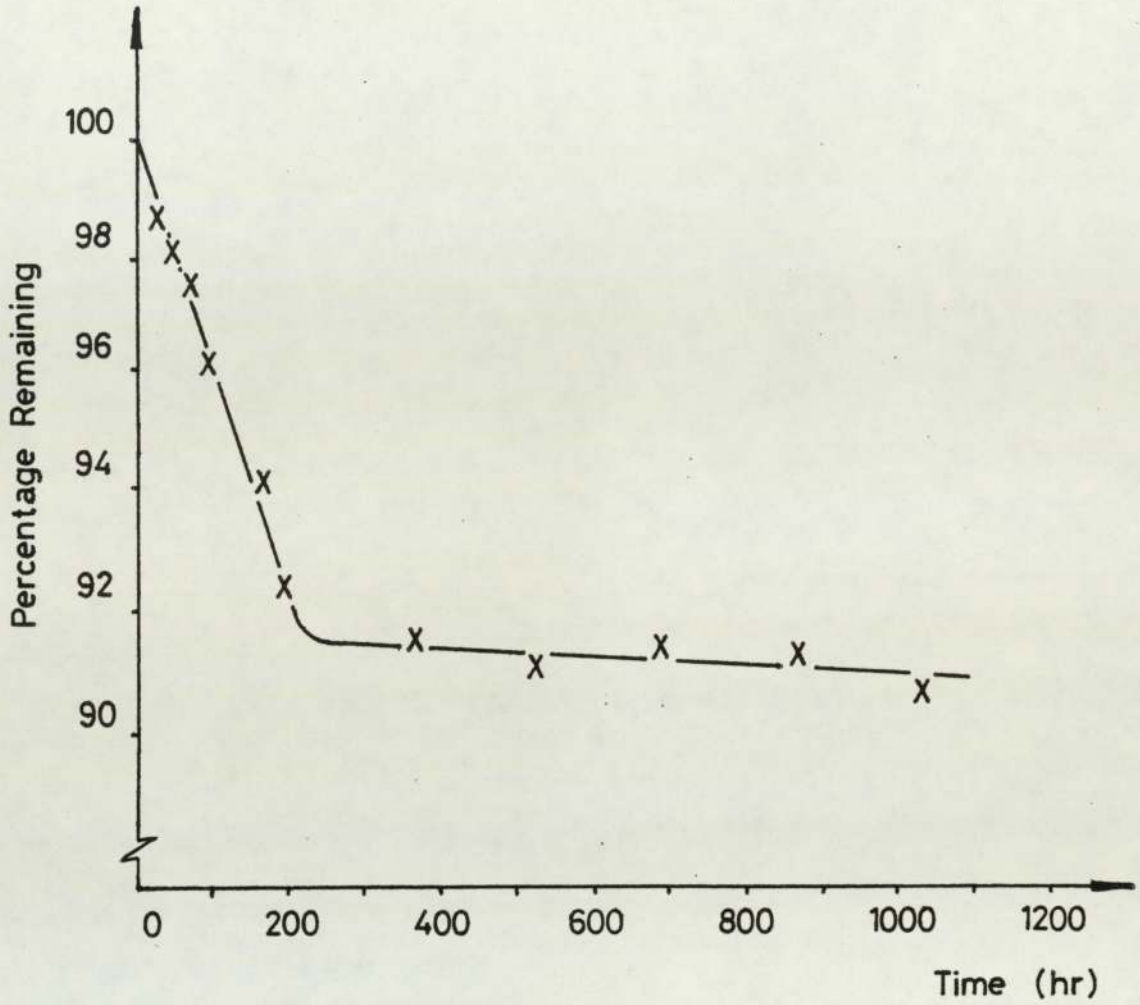


Figure 5.1. Natural logarithm of the percentage doxorubicin remaining versus time in PVC minibags in sodium chloride 0.9%, pH 6.47 at 4°C.

Doxorubicin has been reported to adsorb onto glass and certain plastics [182,183]. This phenomenon may explain the biphasic degradation pattern observed for this and the other studied drugs. In order to investigate this hypothesis the disappearance of doxorubicin from glass containers, approximately 100µg/ml, in the presence of an excess of coarsely chopped PVC (8.0g) was compared to drug loss in

identical control solutions, at 25°C. Figure 5.2 shows a plot of the percentage doxorubicin remaining versus time for these solutions. This plot shows that drug loss during the first 24 hours was much more rapid in the vials which contained chopped plastic than in the controls. The terminal slope of both plots was similar. These data suggest that the initial, rapid, decrease in drug concentration was due to adsorptive losses onto the plastic.

A comparison of the initial drug loss from solutions in glass vials (approximately 100µg/ml) with loss from identical solutions stored in 100ml PVC minibags showed that although the initial rate of adsorption onto glass was slightly greater than that onto PVC the rate constants (calculated from the terminal slope) at 4°C were similar (9.40×10^{-5} hr^{-1} and 8.19×10^{-5} hr^{-1}) respectively.

Drug loss due to adsorption is greater (as a proportion of the total amount) at lower drug concentrations. To investigate this hypothesis the rate of disappearance of doxorubicin, in PVC minibags in sodium chloride 0.9%, pH 5.2, was investigated at concentrations of 50, 100 and 200µg/ml. The rate constants for drug loss in these solutions are shown in Table 5.1. These results show that loss of doxorubicin was greater at 50µg/ml than at 100µg/ml and lowest at 200µg/ml. These data also suggest that the initial slope of the first order plot was due to adsorption onto the container. For this reason rate constants for solutions which were approximately 100µg/ml, were calculated from the terminal slope of the first order plots.

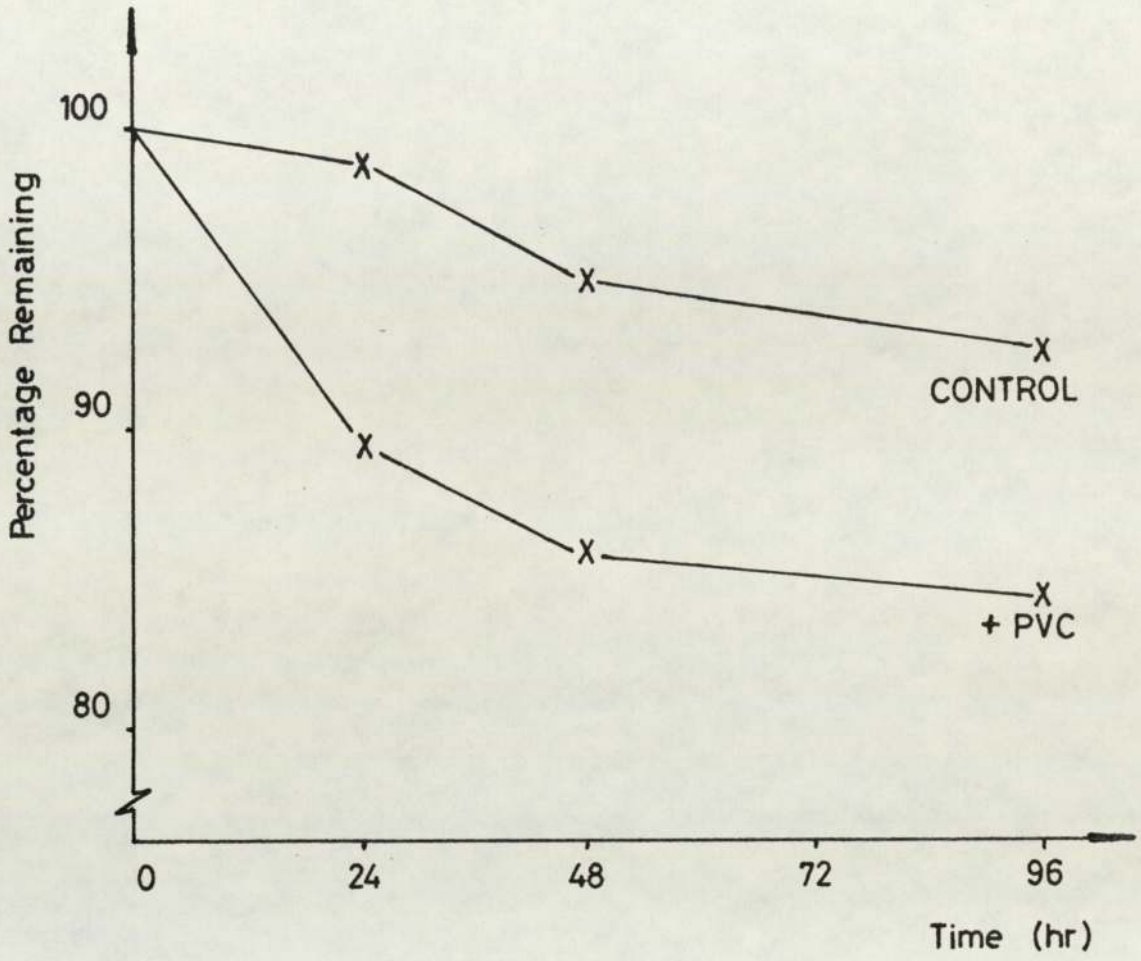


Figure 5.2. Percentage doxorubicin remaining versus time in glass vials (with or without an excess of chopped PVC (8.0g) at 25°C.

CONCENTRATION (μg/ml)	RATE CONSTANT (hr^{-1})
200	1.37×10^{-4}
100	3.37×10^{-4}
50	4.72×10^{-4}

Table 5.1. Rate constants (initial slope) for loss of doxorubicin in sodium chloride 0.9%, pH 5.2, in PVC minibags at 4°C.

A review of the literature revealed that the disappearance of doxorubicin was expected to follow first order kinetics [174,175,177]. However, in those studies the disappearance of doxorubicin was investigated at 1.4mg/ml, 2mg/ml and 2mg/ml respectively. At such high concentrations drug loss due to adsorption is negligible (as a proportion of the total amount) and therefore a biphasic degradation pattern would not be expected. Benvenuto et al [172] and Poochikian et al [173] studied the degradation of doxorubicin in glass containers at 180µg/ml and 10µg/ml respectively. Although adsorption of doxorubicin onto the containers was mentioned by these authors the first order plots did not show a biphasic degradation pattern.

Drug loss due to adsorption is usually complete within the first few hours of the solution coming into contact with the container as theoretically this phenomenon ceases upon formation of a monolayer of the adsorbed drug. The data in this experiment suggest that "adsorption" appeared to continue for up to eight days therefore it is possible that a rapid adsorption onto the container occurred initially, followed by a slower dissolution and migration of the drugs into the plastic matrix. This sort of model has been suggested for the interaction of glyceryl trinitrate, warfarin and some of the benzodiazepines with PVC [195,196,197]. Therefore, as the mechanism of interaction of doxorubicin, daunorubicin and epirubicin with the container appeared to involve more than one process the term "sorption" is used to denote binding of these drugs to the

container in the rest of this section.

The amount of drug sorbed to a container depends on many factors including the vehicle, the surface-area to volume ratio of the filled container, the drug concentration, the time of exposure and the temperature. The interaction of the drug with the container also depends on the drug and the materials from which the container is made [198].

The unionised species of the drug, which is more lipophilic than the ionised species, is expected to be preferentially sorbed to the plastic. The relative amount of the unionised form is controlled by the pH of the solvent and the pKa of the drug. Doxorubicin, daunorubicin and epirubicin have pKa values of about 8.4, 8.3 and 8.0 respectively (Table 1.3) therefore as the pH of the solvent increases a larger percentage of these drugs will be unionised and available for interaction with the plastic. This relationship was clearly shown in a comparison of the drug loss from PVC minibags, which were stored at 25^{o}C, over the first 8 days of the study. Results showed that the amount of drug sorbed from sodium chloride 0.9%, pH 6.47, was greater than from sodium chloride 0.9%, pH 5.2, and was lowest from dextrose 5%, pH 4.36 (Table 5.2).

DRUG	PERCENTAGE LOSS DUE TO SORPTION								
	Dextrose 5% pH 4.36			NaCl 0.9% pH 5.2			NaCl 0.9% pH 6.47		
	25°C	4°C	-20°C	25°C	4°C	-20°C	25°C	4°C	-20°C
DOX	5.2	3.0	2.3	7.3	6.8	3.2	7.4	7.7	3.9
EPI	4.4	2.1	3.4	4.9	2.8	2.8	8.6	4.3	4.4
DAU	4.7	3.3	2.7	4.1	4.0	3.3	3.8	3.5	3.1

Table 5.2. Percentage doxorubicin, daunorubicin and epirubicin sorbed to 100ml PVC minibags after 192 hours.

Illum and Bundgaard [195] showed that the apparent partition coefficient K of a compound between plastic and the solution could be estimated using the following equation;

$$K = \frac{\rho V}{\alpha W_p}$$

where V = volume of solution

ρ = density of solution

W_p = weight of plastic

α = ratio of the final concentration to the total concentration drop according to the equation;

$$\alpha = \frac{F_\infty}{1 - F_\infty}$$

where F_∞ = the equilibrium fraction of drug remaining in solution.

The partition coefficients of doxorubicin, daunorubicin and epirubicin between PVC and the solution were calculated using the above equations. The density of the solutions was calculated by dividing the mass of the solution by the volume present in the minibags after addition of the drug. The weight of the plastic in this study was taken as 8.0g (the weight of the minibag minus the additive ports and the flap at the top of each bag) as the solution was not in contact with this portion of the plastic. Illum and Bundgaard [195] used 11g as the weight of plastic for calculation of the partition coefficients of diazepam, warfarin and glyceryl trinitrate. The log K values obtained in the present work for doxorubicin, epirubicin and daunorubicin were 0.1, 0.2 and -0.2 respectively (for 8.0g of PVC in sodium chloride 0.9%, pH 6.47 at 25°C). The values obtained for diazepam, warfarin and glyceryl trinitrate by Illum and Bundgaard were 1.7, 1.9 and 1.6 respectively (for 11g of PVC). The data in these experiments indicate that partition of doxorubicin, daunorubicin and epirubicin into PVC was minimal as the partition coefficients were small compared to the values for diazepam, warfarin and glyceryl trinitrate which were strongly sorbed to PVC [195].

Illum and Bundgaard [195] also reported that the hexane:water partition coefficient was the most useful parameter for the prediction of the interaction of a particular drug with PVC infusion bags. The data in this experiment also indicate that the butanol:water partition coefficients (16.2, 6.4 and 32.3 for doxorubicin,

daunorubicin and epirubicin respectively) were also a useful predictor of the sorption of these anthracyclines to PVC.

Illum and Bundgaard [195] also observed that a greater fractional uptake of diazepam occurred from 100ml bags than from 500ml bags which resulted from an increase in the surface-area to volume ratio as the volume of solution in the bag decreased. In this experiment the effect of the surface-area to volume ratio was examined by comparison of the amount sorbed to 100ml PVC minibags with the amount sorbed to squares of PVC (10mm x 10mm) which were stored in glass containers. After 192 hours drug loss due to sorption from a solution of doxorubicin (approximately 100µg/ml) in Water For Injections stored in a 100ml PVC minibag was 5.8%. Sorption from an identical solution containing 8.0g of the PVC squares was 14%. The surface area of the PVC squares was calculated as 2.15 times that of the 100ml minibag when the thickness of the bags was taken into account (381µm ± 38) [199]. If drug loss due to adsorption onto the glass container in which the strips were placed is taken into account (approximately 1.5% over the first 24 hours as shown in Figure 5.2) then it can be concluded that drug loss due to sorption to PVC was directly proportional to the surface area of the PVC.

A comparsion of the effect of temperature on the amount of drug sorbed to 100ml PVC minibags showed that as the storage temperature decreased the amount of drug sorbed to PVC decreased. Drug loss in solutions which were frozen and stored at -20°C probably occurred during the time taken for the solutions to freeze after preparation and subsequently

when the drug was thawed and re-frozen after sampling.

In summary, the overall model for the sorption process discussed above is probably most accurately described by an initial rapid adsorptive phase, which appeared to play only a minor role in the overall loss of drug, followed by a slow dissolution and migration of the drug into the plastic matrix. For doxorubicin, daunorubicin and epirubicin the amount of drug lost by sorption to the PVC minibags in this experiment was not significant (less than 10%) at the end of the eight days which were required for equilibration of these drugs with the plastic.

Tables 5.3, 5.4 and 5.5 show the rate constants, calculated from the terminal slope, for disappearance of doxorubicin, epirubicin and daunorubicin respectively in each infusion fluid tested and the percentage of each drug remaining after storage in PVC minibags for 6 weeks. These data show that all three drugs were more stable when refrigerated at $4^{o}C$, or frozen at $-20^{o}C$, than at room temperature ($25^{o}C$).

Results from a two-way analysis of variance (with replicates) for the data in Tables 5.3, 5.4 and 5.5 are shown in Tables 5.6, 5.7 and 5.8 respectively. The F value from Tables [180] at the 5% level of significance for D.F. (2 , 27) for the columns which were concerned with the effect of the temperature was 3.35. As this value was smaller than the calculated F-ratio for each drug (8.62, 42.19, 7.35 for doxorubicin, epirubicin and daunorubicin respectively) this indicated that the storage temperature

significantly affected the percentage of drug remaining at the end of the experiment.

Statistical analysis (Student's t Test) of repeated freezing and thawing showed that eleven freeze-thaw cycles of each drug, at ambient temperature, did not result in a significantly greater loss of drug when compared to control solutions which were frozen and re-thawed only twice over a similar period ($p > 0.05$) (Table 5.9). Hoffman et al [177] made similar observations for solutions of doxorubicin which were frozen and re-thawed six times at ambient temperature.

The effects of thawing by microwave radiation have also been studied. Karlsen et al [174] observed that the concentration of doxorubicin, in PVC minibags, declined significantly after four re-thawings in a microwave ($p < 0.05$). Keusters et al [175] compared the effects of freezing and re-thawing solutions in PVC minibags at ambient temperature and by microwave radiation. Results showed that doxorubicin was stable for two weeks if stored at -20°C and thawed by either method. When these solutions were re-frozen, thawed and assayed five weeks later a small, but significant (t values not quoted), difference in drug concentration was observed in solutions thawed by both methods ($p<0.05$). These authors did not conclude whether storage time or repeated freezing and thawing had caused the observed decrease in concentration.

The statistical analysis on the data published by Keusters et al [175] was re-examined in order to estimate the t values calculated by these authors, using the same method of calculation of pooled standard deviation and

standard error of the difference of the two sample means used in this study. The following results were obtained. A comparison of solutions stored at -20°C for two weeks and thawed at room temperature with identical solutions thawed in a microwave yielded a t value of 0.6787. The value from Tables [180] was 2.306 therefore the difference between the means was not significant ($p > 0.05$). However a comparsion of solutions stored for five weeks and thawed either at room temperature or in a microwave yielded t values of 2.176 and 3.969 respectively. The t value from Tables was 2.306 therefore these data indicated that the decrease in doxorubicin concentration was not significant ($p > 0.05$) if thawed at room temperature but was significant for solutions thawed in a microwave. Therefore the data obtained by Keusters et al [175] appear to be in agreement with the data from this study which indicated that solutions of doxorubicin, daunorubicin and epirubicin were stable when frozen and stored at -20°C for at least five weeks if thawed at room temperature. Solutions could also be thawed and re-frozen several times, at room temperature, without a statistically significant decrease in concentration.

Solutions stored at -20°C take four to five hours to re-equilibrate to room temperature if not artificially heated whereas a single 100ml minibag (frozen at -20° C) takes about two minutes to reach room temperature when exposed to microwave radiation. However, as stated by Ausman et al [200] there are several levels of microwave energy emitted by commercial and domestic appliances which can

produce different magnitudes of heating for the same exposure time. Many are not designed to distribute energy evenly and local "hot-spots" can develop which can result in overheating of the solution and subsequent degradation. It is possible that the increased drug loss observed by Keusters et al [175] and Karlsen et al [174] after thawing in a microwave was due to this phenomenon.

Aglycone degradation products are potentially more cardiotoxic than the parent anthracyclines and, therefore, care must be taken to avoid overheating of solutions and subsequent degradation. As there was no statistically significant difference ($p > 0.05$) between solutions which were refrigerated and those which were frozen storage of these anthracyclines in PVC minibags ,at 4°C, may be more convenient.

Tables 5.3, 5.4 and 5.5 also show that doxorubicin and epirubicin were chemically stable (less than 10% drug loss) in dextrose 5%, pH 4.36, and sodium chloride 0.9%, pH 5.2, for 6 weeks at all temperatures studied. In sodium chloride, pH 6.47, significant loss of both analogues occurred in solutions stored at 25°C over the same period. The t90% values, calculated graphically from the first order plots, were 24 days and 20 days respectively for doxorubicin and epirubicin. Conversely, daunorubicin was chemically stable, in all three infusion fluids studied, for 6 weeks, at 25°C, 4°C and -20°C.

INFUSION FLUID	PH	TEMP. ($^{\circ}$C)	% REMAINING ($\bar{x} \pm$ SD) (n = 4)	RATE CONSTANT (hr^{-1})
Dextrose 5%	4.36	25	92.8 ± 0.5	2.33×10^{-5}
		4	96.3 ± 2.2	1.40×10^{-5}
		-20	96.3 ± 0.5	1.21×10^{-5}
NaCl 0.9%	5.20	25	90.4 ± 1.4	3.23×10^{-5}
		4	92.2 ± 1.0	1.45×10^{-5}
		-20	95.4 ± 0.6	1.21×10^{-5}
NaCl 0.9%	6.47	25	89.1 ± 0.6	4.69×10^{-5}
		4	89.6 ± 1.7	2.38×10^{-5}
		-20	93.7 ± 2.8	2.64×10^{-5}

Table 5.3. Rate constants for drug loss and percentage doxorubicin remaining after storage for 6 weeks in dextrose 5% and sodium chloride 0.9% in PVC minibags.

INFUSION FLUID	PH	TEMP. ($^{\circ}$C)	% REMAINING ($\bar{x} \pm$ SD) (n = 4)	RATE CONSTANT (hr^{-1})
Dextrose 5%	4.36	25	93.9 ± 0.8	2.46×10^{-5}
		4	95.9 ± 0.6	1.73×10^{-5}
		-20	96.4 ± 1.9	1.33×10^{-5}
NaCl 0.9%	5.20	25	92.3 ± 1.8	3.73×10^{-5}
		4	95.5 ± 1.0	2.10×10^{-5}
		-20	96.2 ± 1.4	1.28×10^{-5}
NaCl 0.9%	6.47	25	87.1 ± 2.0	5.25×10^{-5}
		4	93.3 ± 1.0	3.07×10^{-5}
		-20	94.1 ± 0.5	2.18×10^{-5}

Table 5.4. Rate constants for drug loss and percentage epirubicin remaining after storage for 6 weeks in dextrose 5% and sodium chloride 0.9% in PVC minibags.

INFUSION FLUID	PH	TEMP. ($^{\circ}$C)	% REMAINING ($\bar{x} \pm$ SD) (n = 4)	RATE CONSTANT (hr^{-1})
Dextrose 5%	4.36	25	93.3 ± 1.6	1.81×10^{-5}
		4	95.6 ± 1.3	1.76×10^{-5}
		-20	96.5 ± 0.6	1.14×10^{-5}
NaCl 0.9%	5.20	25	93.9 ± 1.2	3.01×10^{-5}
		4	94.5 ± 1.3	1.91×10^{-5}
		-20	96.1 ± 1.3	1.35×10^{-5}
NaCl 0.9%	6.47	25	94.1 ± 1.4	2.58×10^{-5}
		4	95.6 ± 1.1	1.47×10^{-5}
		-20	95.4 ± 1.9	1.48×10^{-5}

Table 5.5. Rate constants for drug loss and percentage daunorubicin remaining after storage for 6 weeks in dextrose 5% and sodium chloride 0.9% in PVC minibags.

SOURCE	SUM SQUARES	D.F.
Rows	163	2
Columns	58.3125	2
Interactions	12.625	4
Residuals	91.3125	27
Total	325.25	35

SOURCE	MEAN SQUARE	F-RATIO (DF1,DF2)
Rows	81.5	24.09856 (2 , 27)
Columns	29.15625	8.62115 (2 , 27)
Interactions	3.15625	0.9332649 (4 , 27)
Residuals	3.381945	

Table 5.6. Two-way analysis of variance (with replicates) for doxorubicin in dextrose, pH 4.36, and sodium chloride 0.9% pH 5.2 and 6.47.

Key:

Rows = The effect of the pH of the infusion fluid.
Columns = The effect of the storage temperature.

SOURCE	SUM SQUARES	D.F.
Rows	82.03125	2
Columns	123.0625	2
Interactions	15.875	4
Residuals	39.375	27
Total	260.3438	35

SOURCE	MEAN SQUARE	F-RATIO (DF1,DF2)
Rows	41.01563	28.125 (2 , 27)
Columns	61.53125	42.19286 (2 , 27)
Interactions	3.96875	2.721429 (4 , 27)
Residuals	1.458333	

Table 5.7. Two-way analysis of variance (with replicates) for epirubicin in dextrose 5%, pH 4.36, and sodium chloride 0.9% pH 5.2 and 6.47.

Key:

Rows = The effect of the pH of the infusion fluid.
Columns = The effect of the storage temperature.

SOURCE	SUM SQUARES	D.F.
Rows	0.3125	2
Columns	26.625	2
Interactions	10.03125	4
Residuals	48.875	27
Total	85.84375	35

SOURCE	MEAN SQUARE	F-RATIO (DF1,DF2)
Rows	0.15625	0.086317 (2 , 27)
Columns	13.3125	7.35422 (2 , 27)
Interactions	2.507813	1.38539 (4 , 27)
Residuals	1.810185	

Table 5.8. Two-way analysis of variance (with replicates) for daunorubicin in dextrose 5%, pH 4.36, and sodium chloride 0.9% pH 5.2 and 6.47.

Key:

Rows = The effect of the pH of the infusion fluid.
Columns = The effect of the storage temperature.

DRUG	PERCENTAGE REMAINING AFTER TWO RE-THAWINGS $\bar{x} \pm$ SD (n = 3)	PERCENTAGE REMAINING AFTER ELEVEN RE-THAWINGS $\bar{x} \pm$ SD (n = 3)
DOX	99.1 ± 2.0	96.7 ± 1.8
EPI	97.5 ± 0.7	95.6 ± 1.4
DAU	99.3 ± 0.9	97.6 ± 1.5

Table 5.9. Percentage doxorubicin, daunorubicin and epirubicin remaining after two freeze-thaw cycles and after eleven freeze-thaw cycles (at ambient temperature in dextrose 5% in PVC minibags over a 4 week period.

A comparison of the rate constants for degradation of each drug in half strength Britton-Robinson buffer, pH 4.35, pH 5.33 and pH 6.37 with those for drug loss in identical solutions reconstituted with dextrose 5%, pH 4.36, sodium chloride 0.9%, pH 5.2, and sodium chloride 0.9%, pH 6.47, showed that the rate of degradation in aqueous buffer solutions was much more rapid than in infusion fluids of almost identical pH values (Table 4.1 and 5.3, 5.4 and 5.5 respectively). These and other experimental data showed that at constant temperature, pH and ionic strength the rate of degradation of these drugs was increased in the presence of buffers. Buffer catalysis was also shown in recent experiments by Beijnen *et al*. These authors observed a non-linear buffer catalysis of daunorubicin between pH 3.0 and 11.5 [185] and of doxorubicin at pH values less than or equal to pH 9.5 [184].

The only structural difference between doxorubicin and

daunorubicin is the substituent on the C14 atom in the aglycone portion of the molecule (a proton in daunorubicin and a hydroxyl group in doxorubicin). Beijnen et al [176] postulated that this structural difference must hold the key to the differences in stability observed for these two analogues in sodium chloride 0.9%, pH 6.47. The increased rate of degradation of doxorubicin and epirubicin may be explained by the fact that the C-9 side chain present in these analogues undergoes enolisation in weakly alkaline solution as described in section 4.3.ii. Enolisation results in anion production and therefore may lead to an increase in the rate of oxidation of doxorubicin and epirubicin compared to daunorubicin. Enolisation also promotes formation of 7,8-dehydro-9,10-desacetyldaunorubicinone rather than the bis-anhydro derivatives that are also formed during the degradation of daunorubicin in alkaline solution. These data suggest that in alkaline solution the mechanism of degradation of doxorubicin and epirubicin is different to the mechanism of degradation of daunorubicin.

In a recent study of the stability of these anthracyclines (100µg/ml) in polypropylene tubes at ambient temperature (25° C) Beijnen et al [176] observed that daunorubicin was stable for 4 weeks in both dextrose and sodium chloride-containing admixtures. These authors also observed that doxorubicin and epirubicin were stable in dextrose 5%, pH 4.7, and a mixture of dextrose 3.3% with sodium chloride 0.3%, pH 4.4, but significant degradation was observed in sodium chloride 0.9%, pH 7.0. The t90%

values, obtained by these authors, for doxorubicin and epirubicin were 6 days and 8 days respectively. The difference between the t90% values quoted by Beijnen et al [176] and those obtained for sodium chloride 0.9%, pH 6.47 in this experiment, may be partially explained by the difference in pH of the infusion fluids studied. A comparison of the rate of degradation of these two analogues in buffers at pH values greater than 5.0 showed a rapid increase in the rate of degradation with increasing pH (Figure 4.20 and 4.21). If a similar increase in the rate of drug loss is observed as the pH of the drug infusion fluid admixture increases then the time for 10% degradation to occur (t90% value) would be expected to decrease as the pH of the infusion fluid increased.

Doxorubicin and epirubicin became progressively more stable as the pH of the drug infusion fluid admixture became more acidic (pH 6.47 to pH 4.36) and were optimally stable in dextrose 5%, pH 4.36. A review of the literature revealed that Poochikian et al [173] made similar observations for doxorubicin.

Results from the two-way analysis of variance (with replicates) (Tables 5.6, 5.7 and 5.8) showed the following results. The F value, at the 5% level of significance (for DF 2,27) for the rows, which represented the effect of the type of infusion fluid, was 3.35. The values for the calculated F-ratios were 0.863, 28.125 and 24.098 for daunorubicin, epirubicin and doxorubicin respectively. As the F values for doxorubicin and epirubicin were very much larger than 3.35 the data indicate that the difference in

the percentage of doxorubicin and epirubicin remaining at the end of the study in the three infusion fluids was highly significant and that the pH of the infusion fluid used to reconstitute these two analogues was extremely important. As the calculated F-ratio for daunorubicin was much smaller than 3.35 the percentage daunorubicin remaining at pH 4.36, 5.2 and 6.47 was not significantly different which indicated that the stability of daunorubicin did not appear to be affected by the pH of the infusion fluid.

Results from a three-way analysis of variance (with replicates) (Table 5.10) confirm that Factors 1, 2 and 3 (drug, temperature and pH effects respectively) alone strongly influenced the rate of degradation of these anthracyclines whereas the interactions between these variables were only of minor importance.

These data suggest that solutions of doxorubicin and epirubicin (approximately 100μg/ml) may be stored in dextrose 5% or sodium chloride 0.9%, for at least 6 weeks, providing that the pH of the sodium chloride is at the lower end of the pharmacopoeial limits (the pH of sodium chloride 0.9% should be within the range pH 5.0 to 7.0). Prolonged storage of low concentrations of doxorubicin or epirubicin in solutions of sodium chloride 0.9%, with pH values at the upper end of this pH range, is not recommended unless the solutions are frozen and stored at -20 °C. Conversely, it appears that daunorubicin may be stored, for at least 6 weeks, in either dextrose 5%, 4.36, or sodium chloride 0.9% solution between pH 5.0 and 7.0.

SOURCE	SUM OF SQUARES	D.F	MEAN SQUARE	F
FACTOR 1	157.125	2	78.5625	35.42655
FACTOR 2	149.9375	2	74.96875	33.806
FACTOR 3	187.9375	2	93.96875	42.37376
1X2	95.4375	4	23.85938	10.75902
1X3	20.0625	4	5.015625	2.261719
2X3	2.3125	4	0.578125	0.260696
1X2X3	36.0625	8	4.507813	2.032728
ERROR	179.627	81	2.217617	
TOTAL	828.5625	107		

Table 5.10. Three-way analysis of variance (with replicates) for doxorubicin, daunorubicin and epirubicin in dextrose 5%, pH 4.36 and sodium chloride 0.9% pH 5.2 and 6.47.

Key:

FACTOR 1 = The effect of the drug.

FACTOR 2 = The effect of the pH of the infusion fluid.

FACTOR 3 = The effect of the storage temperature.

A comparison of these data with the data obtained from the pH profiles (chapter four) showed the following results. The pH profiles for epirubicin and doxorubicin (Figures 4.20 and 4.21 respectively) showed optimum stability for these two analogues between pH 4.0 and 5.0. As the pH of the drug-buffer admixture increased between pH 5.0 and pH 7.2 a rapid increase in the rate of drug loss was observed. These data predict the increased rate of disappearance of these two analogues which was observed as the pH of the infusion fluids increased. Conversely, the pH profile for daunorubicin (Figure 4.22) showed very little change in the rate constant for degradation in buffers between pH 5.0 and pH 7.2 and consequently daunorubicin appeared to be almost equally stable in either dextrose 5% or sodium chloride 0.9%.

These drugs are usually administered within a few hours of preparation. At the higher concentrations which are normally used in cancer chemotherapy (greater than or equal to 500μg/ml) drug loss due to adsorption onto the container and penetration of these drugs into the plastic matrix are small (as a proportion of the total amount of drug present). In addition, the differences in stability which were observed in this experiment for different infusion fluids is probably not significant. Therefore in the practical situation, reconstituted solutions of doxorubicin, epirubicin or daunorubicin may safely be added to either dextrose 5% , sodium chloride 0.9% or dextrose/saline.

The pH of both the dextrose- and sodium chloride-

containing admixtures increased by approximately half a unit (4.4 to 4.9, 5.3 to 6.0, 6.5 to 7.1 for dextrose and the two sodium chloride solutions respectively) over the period of study. Benvenuto et al [172] also observed a pH increase (4.75 to 6.26) which was accompanied by a colour change of the doxorubicin admixtures from orange to reddish orange. However, the data presented by these authors was rather misleading as tabulated pH values were quoted for sodium chloride 0.9% and stability in dextrose 5% was quoted in the text. Conversely, Beijnen et al [176] observed a slight decrease in the pH of dextrose-containing admixtures. No significant change in the pH of sodium chloride 0.9% admixtures was recorded, by these authors, but a red-coloured precipitate accompanied degradation in those solutions. In this experiment no colour change or precipitation was observed in any of the solutions.

After completion of this study the minibags were emptied. The inside surface showed a dull pink coating which confirmed that either parent drug or degradation products had sorbed to the container. Beijnen et al [185] observed that, at pH values greater than 4, aglycone degradation products such as DrVIII (Figure 4.14) and the bis-anhydro derivatives, if formed, were strongly hydrophobic and adsorbed onto silanised glass showing a dull pink coating. These products probably also sorb to PVC and may therefore, be responsible for the dull pink coating observed on the minibags. Conversely, in containers where adsorption is known not to occur, such as polypropylene, these aglycones precipitate. This possibly explains why Beijnen et al [176]

observed precipitation during degradation of doxorubicin and epirubicin in polypropylene tubes.

Chromatograms showed little evidence of degradation products despite the fact that significant drug losses (greater than or equal to 10 %) were observed in some of the admixtures tested. Poochikian et al [173] also made a similar observation and suggested that the degradation products either had a weaker chromophore or that the λ max values were shifted to other wavelengths. After consideration of the experimental and literature data and the lipophillic nature of the aglycones it is likely that the lack of degradation products on chromatograms in this experiment is most readily explained by their sorption to the container in the same way as the unionised form of the parent compound sorbed to the plastic.

Degradation products were observed in Sorenson buffer pH 4.0, after storage at 25^{o}C for 168 hours in glass containers. These data predict that formation of degradation products in infusion fluids of similar pH values is likely to occur. As indicated earlier in this section the rate of degradation of doxorubicin is accelerated in the presence of buffers but the data suggest that it may be possible to detect degradation products in infusion fluids if the solutions were stored for long enough periods.

5.3.ii. Stability in polypropylene syringes.

Results from preliminary experiments with low

concentrations of drug (approximately 100μg/ml) showed that loss of doxorubicin in polypropylene syringes, at 4°C and 25° C, was more rapid than from solutions of identical concentration which were stored in PVC minibags (Tables 5.11 and 5.3 respectively). Doxorubicin has been reported not to sorb to polypropylene [182]. This phemonenon was explained by Illum and Bundgaard [195] to be due to the fact that polypropylene does not contain plasticizers whereas the plasticizer present in PVC, di-2-ethylhexyl phthalate, was suggested to be of major importance for the sorption of chemicals to PVC. Therefore another explanation for the increased rate of drug loss in these syringes was sought. It was hypothesized that doxorubicin adsorbed onto the rubber on the end of the syringe plunger, and/or that contact of the solution with this rubber, increased the rate of drug loss. In order to investigate this hypothesis the rate of disappearance of doxorubicin, in glass vials in the presence of an excess of coarsely chopped rubber (7.3g) was compared to drug loss in identical control solutions.

The pH of the admixture which contained the chopped rubber increased by approximately 1 unit during the study. This pH change was accompanied by formation of a red-coloured precipitate which necessitated filtration of these solutions through a 0.22μm filter (Millex-OR Millipore) immediately prior to injection onto the HPLC. Chromatograms of these admixtures showed several other peaks in addition to the parent compound peak (Figure 5.3) which may be due to degradation products or leaching of compounds from the rubber.

The first order plots for disappearance of doxorubicin in the presence of excess rubber also showed a biphasic degradation pattern (Figure 5.4). The rate of loss of doxorubicin in the presence of the rubber was also much more rapid than in identical control solutions (Table 5.12). These data suggest that adsorption of doxorubicin onto the rubber probably occurred initially, followed by degradation/loss which may have been catalysed by the presence of the rubber.

The syringe plunger was autoclaved and coated with a silicone lubricant during production. Autoclaving tends to affect the surface of the rubber such that the likelihood of products leaching from the plunger are greatly reduced [201]. However, in this experiment the rubber was cut into quarters and as a result the inside surface was exposed to the drug in solution. For this reason it is likely that the peaks shown in Figure 5.3 are due to leaching of compounds from the rubber.

The effect of contact of the drug solution with the rubber plunger may or may not be important in the clinical situation where doxorubicin, daunorubicin and epirubicin are used in syringes at concentrations of at least 2mg/ml. In order to investigate this hypothesis studies were expanded to include an assessment of the stability of these agents in polypropylene syringes at this concentration.

Thawing of solutions which were frozen at -20° C, in 60ml syringes, was a slow and cumbersome process. A review of the literature revealed that Kirk et al [202] made the

same observation during stability studies with cyclophosphamide. These authors observed that during the freezing process the plunger of the syringe contracted and a small amount of drug solution leaked past the barrel. Although the resultant drug loss was negligible the risk of microbial contamination was markedly increased. For these reasons, studies on the freezing of doxorubicin, daunorubicin and epirubicin in syringes was abandoned in favour of stability studies at 4°C as this was considered to be the most practical storage temperature for these drugs in polypropylene syringes.

TEMP. °C	% REMAINING $\bar{x} \pm SD$ (n = 3)	RATE CONSTANT (hr^{-1})
25	84.6 ± 1.4	3.60×10^{-4}
4	90.9 ± 0.8	1.66×10^{-4}

Table 5.11. Rate constants for drug loss and percentage doxorubicin (100µg/ml) remaining after storage in polypropylene syringes for 4 weeks at 25°C or 4°C.

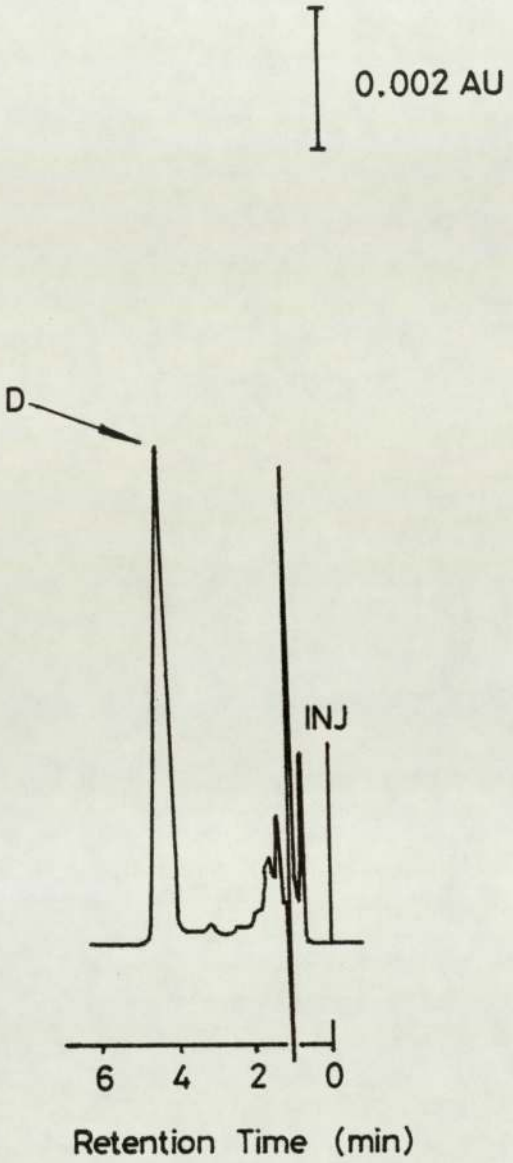


Figure 5.3. High performance liquid chromatogram for doxorubicin after exposure to an excess (7.3g) of coarsely chopped rubber in glass vials for 4 weeks at 25° C. Mobile phase: acetonitrile: water 40:60 (vol:vol; pH 2.5), flow rate: 1.4ml/min, detection: 290nm, chart speed 5mm/min., sensitivity 0.02 AUFS, doxorubicin = D.

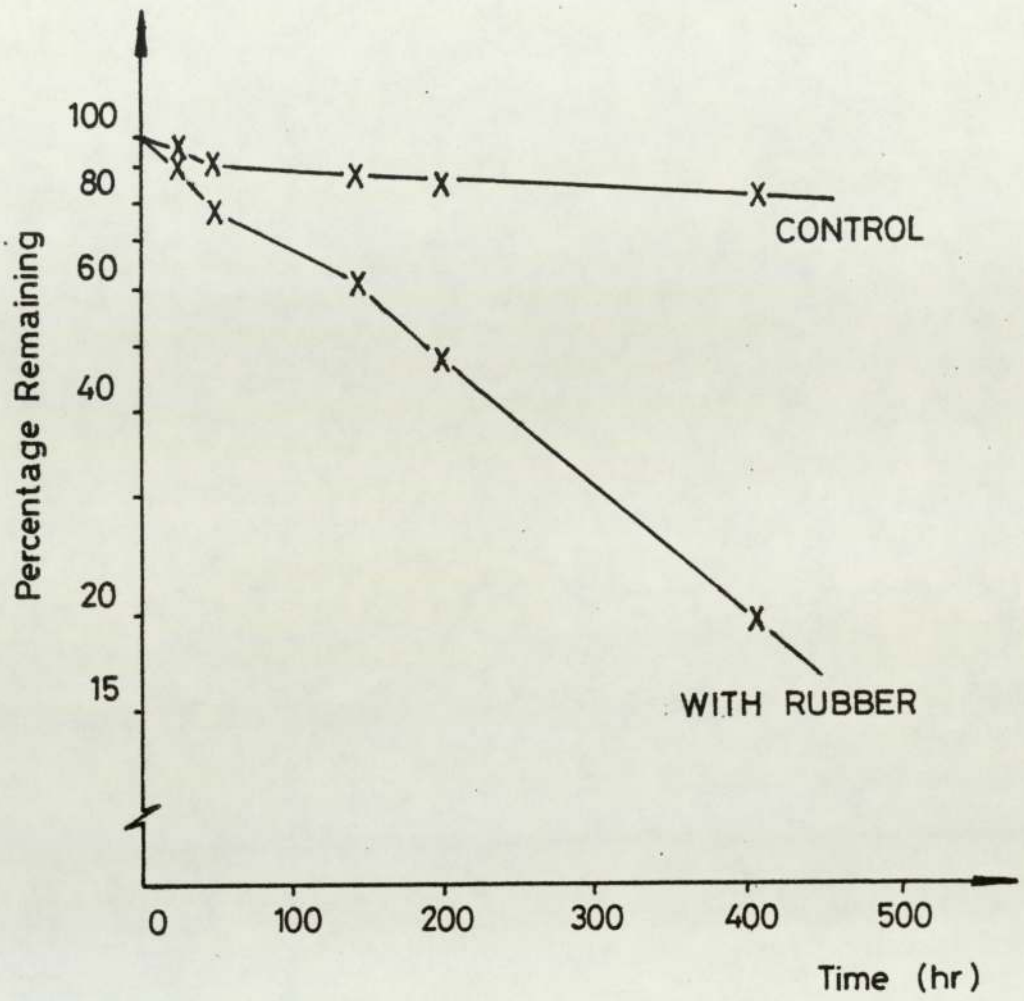


Figure 5.4. Natural logarithm of the percentage doxorubicin remaining versus time in glass vials with and without an excess (7.3g) of chopped rubber at 25°C.

ADMIXTURE	% REMAINING $\bar{x} \pm SD$ (n = 3)	RATE CONSTANT (hr^{-1})
DOX (CONTROL)	87.5 ± 1.9	9.40×10^{-5}
DOX (WITH RUBBER)	-	3.95×10^{-3}

Table 5.12. Rate constants for drug loss and percentage doxorubicin (100µg/ml) remaining after storage in glass vials for 4 weeks at 25°C.

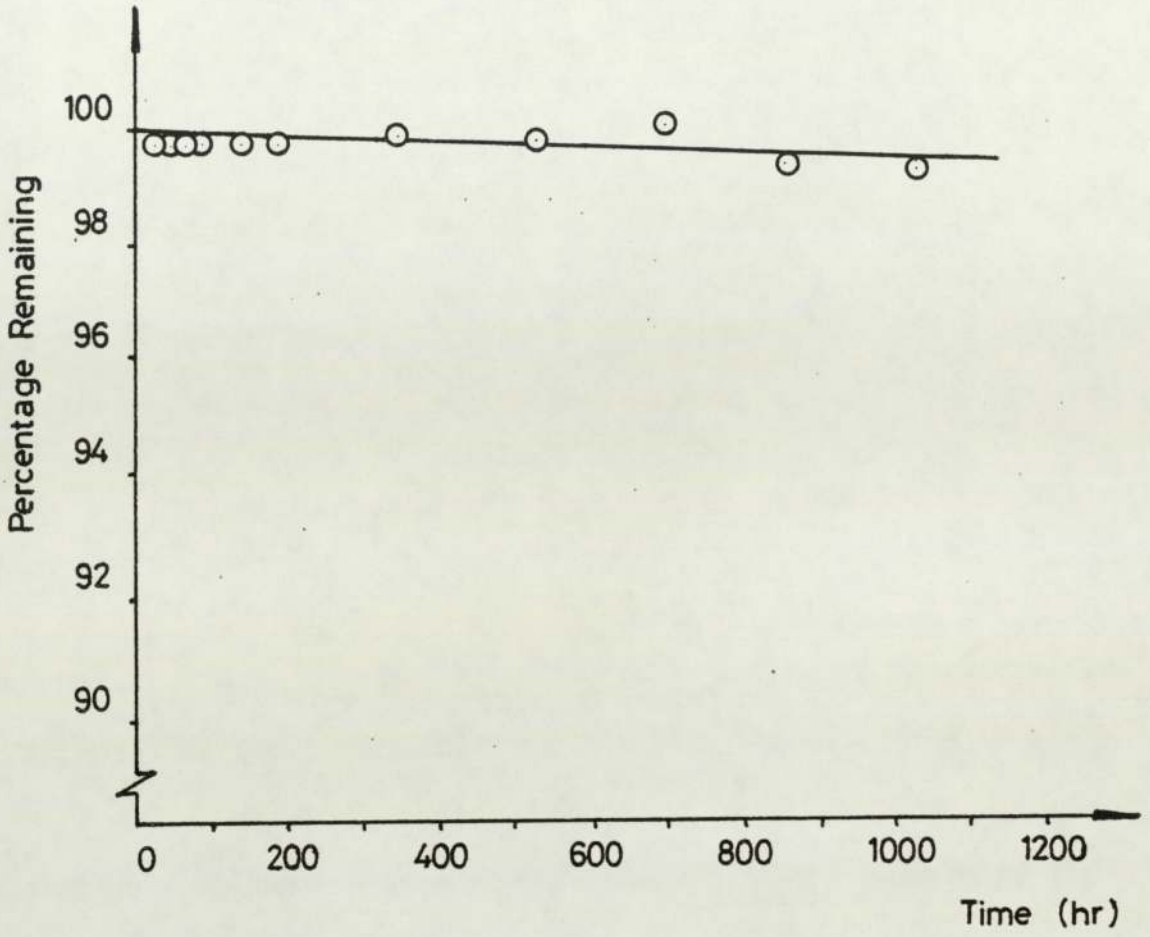


Figure 5.5. Natural logarithm of the percentage epirubicin, 2mg/ml, remaining versus time in polypropylene syringes at 4°C.

DRUG	% REMAINING $\bar{x} \pm$ SD (n = 4)	RATE CONSTANT (hr^{-1})
DOX	98.7 ± 1.0	6.10×10^{-6}
EPI	99.7 ± 0.4	3.30×10^{-6}
DAU	96.9 ± 2.3	2.40×10^{-5}

Table 5.13. Rate constants for drug loss and percentage doxorubicin, daunorubicin and epirubicin (2mg/ml) remaining after storage in polypropylene syringes for 6 weeks at 4°C.

SOURCE	SUM SQUARES	D.F
Columns	12.76563	3
Residuals	23.09375	8
Total	35.85938	11

SOURCE	MEAN SQUARE	F-RATIO (DF1, DF2)
Columns	4.255209	1.474064 (3 , 8)
Residuals	2.886719	

Table 5.14. One-way analysis of variance (with replicates) for doxorubicin, daunorubicin and epirubicin in polypropylene syringes at 4°C.

Table 5.13 shows the rate constants for degradation and the percentage of each drug remaining after storage of a solution (2mg/ml) of each drug in polypropylene syringes for 6 weeks at 4°C. The data show that all three drugs were chemically and physically stable (less than 10% degradation) for at least 6 weeks in polypropylene syringes at this concentration.

Results from a one-way analysis of variance (with replicates) for doxorubicin, daunorubicin and epirubicin in polypropylene syringes at 4°C is shown in Table 5.14. The F

value from Tables (DF 3, 8), at the 5% level of significance, was 4.07. As the calculated F-ratio was much smaller than this value (1.474) these data indicate that the difference between the percentage of each drug remaining at the end of the study was not significant.

Plots of the natural logarithm of the percentage parent drug remaining versus time were linear which indicated that drug loss followed first order kinetics. Figure 5.5 shows such a plot for epirubicin. The linearity of this plot indicated that at a concentration of 2mg/ml drug loss due to adsorption onto the rubber of the syringe plunger was negligible. The data also indicate that contact of these solutions with this rubber during storage did not appear to adversely affect stability as there was no evidence of degradation products on chromatograms. A comparison of the rate of drug loss in polypropylene syringes at 2mg/ml (6.10×10^{-6} hr^{-1}) and at 100µg/ml (1.66×10^{-4} hr^{-1}) at 4°C showed that the rate of disappearance of doxorubicin was approximately twenty five times faster at 100µg/ml than at 2mg/ml. This suggests that the rate of disappearance of doxorubicin was proportional to the reciprocal of the drug concentration and that drug loss was probably due to adsorption onto the rubber syringe plunger because the amount lost was larger (as a proportion of the total amount) in the less concentrated solution.

Comparison of the rate of disappearance of epirubicin, in a polypropylene syringe (3.30×10^{-6} $hour^{-1}$), with the rate of drug loss in an identical solution stored in a glass

vial (3.20×10^{-6} $hour^{-1}$) showed that, at 2mg/ml, epirubicin was equally stable in either glass or polypropylene containers. These data are in agreement with the data presented earlier in section 5.3.i. which showed that the rate constant for drug loss (calculated from the terminal slope) for doxorubicin (approximately 100µg/ml in Water For Injections) was similar in both glass and PVC containers.

In conclusion, at a concentration of 100µg/ml doxorubicin and epirubicin were chemically and physically stable in dextrose 5%, pH 4.36, and sodium chloride 0.9%, pH 5.2, but not in sodium chloride 0.9%, pH 6.47, over the six week period of study. Conversely, daunorubicin was stable in all three infusion fluids. This indicated that structural differences at the 4'-position of the amino sugar moiety appeared to have no influence on the chemical stability of doxorubicin, daunorubicin and epirubicin in dextrose 5% or sodium chloride 0.9%. The substituent on the C14 of the aglycone (a proton in daunorubicin and a hydroxyl in doxorubicin) appeared to affect stability in sodium chloride 0.9%, pH 6.47. These data are in agreement with the results of a study published by Beijnen et al [176] but how this minor structural difference conferred greater stability on daunorubicin in alkaline solution was not indicated by these authors.

The increased rate of degradation of doxorubicin and epirubicin may be explained by the fact that the C-9 side chain present in these analogues undergoes enolisation in weakly alkaline solution as described in section 4.3.ii. Enolisation results in anion production and may therefore

lead to an increase in the rate of oxidation of these two analogues compared to daunorubicin. Enolisation also promotes formation of 7,8-dehydro-9,10-desacetyldaunorubicinone rather than the bis-anhydro derivatives that are also formed during the degradation of daunorubicin in alkaline solution. These data suggest that in alkaline solution the mechanism of degradation of doxorubicin and epirubicin is different to the mechanism of degradation of daunorubicin.

At concentrations of approximately 100μg/ml an initial rapid adsorption of the parent compound onto glass, PVC and the rubber of the syringe plunger was demonstrated but losses were not significant (less than 10%) in this experiment. Drug loss due to adsorption is proportional to the reciprocal of the initial concentration and is therefore particularly important when these drugs are used in low concentrations in the laboratory. In this situation, the type of container used for storage is critical and storage in glass should be avoided.

Drug loss due to adsorption is rapid and theoretically ceases when a monolayer of drug is adsorbed onto the surface of the container. As well as this initial rapid adsorption of drug onto PVC a subsequent migration of the parent compound into the plastic matrix was also observed. As the mechanism of interaction with the container appeared to involve more than one process the overall interaction of these drugs with the container was termed sorption.

Experimental data showed that migration into the PVC

was dependent on the vehicle, the surface-area to volume ratio of the filled container, the drug concentration, the time of exposure and the temperature. The drug container interaction also depends on the drug and the materials from which the container is made [198].

The unionised form of the drug, which is more lipophillic than the ionised species, is expected to be preferentially sorbed to the plastic. The relative amount of unionised drug is controlled by the pKa of the drug and the pH of the solution. Results from this study showed that the amount of drug sorbed to PVC was greater from sodium chloride 0.9%, pH 6.47, than from sodium chloride 0.9%, pH 5.2 and lowest from dextrose 5%, pH 4.36 (Table 5.2).

Illum and Bundgaard [195] indicated that the hexane-water partition coefficient was a useful parameter for the prediction of the interaction of a drug with PVC. The data in this experiment indicated that the butanol:water partition coefficient was also a useful predictor of sorption of the studied anthracyclines to PVC.

The amount of drug sorbed to PVC was also shown to be directly proportional to the surface area of PVC to which the solution was exposed and decreased as the temperature decreased. Sorption in solutions stored at -20°C probably occurred during the time taken for the admixtures to freeze after preparation and subsequently when the solutions were thawed and re-frozen after sampling.

The presence of the plasticizer di-2-ethylhexyl-phthalate has also been reported to be important in the sorption of drugs to PVC. Polypropylene and polyethylene

do not contain plasticizers [195]. Adsorption to polyethylene has been observed [203] but adsorption onto polypropylene has not been reported [182] and was not apparent in this experiment. For this reason it is recommended that solutions of very low concentration should be stored in polypropylene containers.

At a concentration of 2mg/ml all three drugs were chemically and physically stable in both glass and polypropylene containers for at least 2 weeks and 6 weeks respectively. However, as there is no preservative included in the vials of doxorubicin, daunorubicin and epirubicin the manufacturers recommend that after reconstitution these drugs should be discarded after 24 hours at room temperature and 48 hours if refrigerated. The experimental data show that vials of these drugs, reconstituted under aseptic conditions to produce solutions containing approximately 2mg/ml, may be pre-prepared in bulk and stored in a refrigerator for at least 2 weeks. Similarly, the shelf life of syringes, at this concentration, may be extended to several weeks, providing that the pharmacist in charge of the reconstitution service can ensure the sterility of these products at the time of use.

Chapter Six

Conclusion.

In the preceeding chapters stability-indicating HPLC assays were developed for doxorubicin, daunorubicin and epirubicin. Using these assays the degradation kinetics of of these drugs was investigated in fluorescent light. The effect of pH on the rate and mechanism of degradation was investigated by construction of a pH profile for each drug. Finally, the results obtained in these preliminary experiments were applied to a study on the long term stability of these drugs in infusion fluids.

At concentrations such as those used for cancer chemotherapy, (2mg/ml), the experimental results indicated that no special precautions appeared to be necessary to protect freshly prepared solutions of doxorubicin, daunorubicin and epirubicin from either fluorescent light or sunlight. However, Williams and Tritton [167] observed that solutions of doxorubicin (500µg/ml) which were irradiated with UV light at 366nm lost their ability to be cytotoxic to Sarcoma 180 cells *in vitro*. The results of gel filtration studies by those authors showed formation of a new higher molecular weight species, postulated to be a polymer containing ten monomeric units, that could not penetrate the Sarcoma cell. These authors also observed that doxorubicin was inactivated when stored under fluorescent lights in the laboratory. Therefore solutions of doxorubicin and related compounds for therapeutic use should not be exposed to natural or artificial light for prolonged periods.

Photodegradation is of particular importance when these drugs are handled in low concentrations in the laboratory. At concentrations below 100µg/ml drug loss due to

photodegradation may be significant (as a proportion of the total amount) if these drugs are exposed to light for sufficient time. Other authors have observed that photodegradation is directly proportional to the intensity of the light and the distance of the sample away from the source [169]. In this study the room light, which was provided by four 65/80 watt fluorescent tubes mounted approximately one metre above the samples, was considered to be of relatively low intensity. Consequently, the t90% values for solutions of 10µg/ml in clear glass were high (approximately 12 hours, 15 hours and 13 hours for doxorubicin, daunorubicin and epirubicin respectively). These data suggest that drug loss during photodegradation (for solutions in the low nanogram range) may be extremely rapid if the solutions are close to a high intensity light source.

Photodegradation was most rapid in clear glass containers compared to the amber glass and opaque polyethylene which appeared to afford some protection from light. Results from UV spectra indicated that the amber glass containers conformed to the United States Pharmacopoeia (USP) recommendations which state that transmittance should be less than 12% between 290 and 450nm. Therefore these containers gave adequate protection from light. The spectrum obtained for the opaque polyethylene containers suggested that some, but not complete, protection from light was provided by these containers. In summary, photodegradation could be successfully prevented by storage

in amber glass or by covering clear glass or polyethylene containers with aluminium foil. The effect of the type of container use for storage of solutions in the dark is considered later in this chapter.

Other authors have also observed that photodegradation is enhanced in the presence of oxygen [166,185]. The influence of oxygen was not investigated in this study but may be successfully prevented by freshly boiling and cooling the water used for preparation of the solutions and if buffers are used these may be purged with nitrogen. Trace metal ion impurities which may originate from buffers have also been observed to catalyse decomposition [185]. This effect could be prevented by addition of the chelating agent sodium edetate.

Photodegradation was also observed to be dependent on the pH of the solvent and was much more rapid in Tris buffer, pH 7.2 and pH 8.0, than in Water For Injections, pH 6.1. Aglycone degradation products were observed after solutions of doxorubicin (in Tris buffer pH 7.2 and 8.0) were exposed to room light for 8 hours and 2 hours respectively. However, after 96 hours and 48 hours respectively these aglycones were not evident on chromatograms. These data suggested that aglycones were formed initially in these solutions but were broken down by a photobleaching process as proposed by Gray and Phillips [166]. Aglycones were also produced in Tris buffer solutions which were stored in the dark and were not evident in solutions of doxorubicin in Water For Injections, pH 6.1, which suggested that aglycones were not solely

photodegradation products as proposed by Gray and Phillips [166], but were formed as a result of the pH of the solvent and its effect on the rate and mechanism of degradation.

The rate of degradation of doxorubicin, daunorubicin and epirubicin was strongly influenced by the pH of the medium. In strongly acidic solution (pH approximately 1.5) doxorubicin and daunorubicin appeared to degrade at almost the same rate whereas the rate of degradation of epirubicin was substantially slower.

X-ray diffraction studies by Neidle and Taylor [193] and Courseille et al [194] revealed that hydrogen bonding was an important determinant of both the molecular structure and conformation of the crystal structure of the anthracyclines. The high stability of the observed conformation suggested that this conformation was also likely to be favoured in solution. The hydrogen bond mentioned by these authors which is most pertinent to this study was the hydrogen bond exhibited by the positively charged amino group and the C4' hydroxyl. In epirubicin the C4' hydroxyl has an equatorial orientation whereas in doxorubicin the C4' hydroxyl is axial. Therefore this structural difference may lead to a difference in the hydrogen bonding between this hydroxyl and the amino group which may in turn lead to a difference in the distribution of electronic charges in the quinonic chromophore. These changes in charge distribution may have led to a stabilisation of the glycosidic bond in the epirubicin molecule which resulted in greater stability of this

analogue in strongly acidic solution.

In dextrose 5%, pH 4.36, and sodium chloride 0.9%, pH 5.2 and 6.47, in PVC minibags, the rate of disappearance of doxorubicin and epirubicin was similar. Both drugs became progressively more stable as the pH of the drug infusion fluid admixture became more acidic (pH 6.47 to 4.36) and were optimally stable in dextrose 5%, pH 4.36.

Significant loss (greater than or equal to 10%) of both drugs occurred in sodium chloride 0.9%, pH 6.47, at 25°C. The t90% values were 24 and 20 days for doxorubicin and epirubicin respectively.

As both these drugs were almost equally stable in the most acidic of these infusion fluids (dextrose 5% pH 4.36) this suggested that structural differences at the 4' position of the amino sugar moiety appeared to have no influence on the chemical stability of these drugs in this infusion fluid.

A comparison of the rate of degradation of doxorubicin, daunorubicin and epirubicin in buffers showed that the rate of degradation was lowest at pH values of approximately 4.35. Between pH 5.0 and pH 7.2 a rapid increase in the rate of degradation occurred as the pH of the buffer increased (Figure 4.20 and 4.21). These data predict the increased rate of drug loss that was observed for these two analogues in sodium chloride 0.9%, pH 6.47 compared to sodium chloride 0.9%, pH 5.2 and the observation that these two analogues were optimally stable in dextrose, pH 4.36.

Conversely, for daunorubicin the pH profile showed very little increase in the rate of degradation as the pH of the

buffer increased between pH 5.0 and 7.2. These data predict very little difference in the rate of drug loss in sodium chloride 0.9%, pH 6.47, compared to that in sodium chloride 0.9%, pH 5.2, and dextrose 5%, pH 4.36, as was observed in these experiments.

The fact that the rate of degradation of epirubicin in buffers at pH values of approximately 1.5 was significantly altered by the substituent on the C4' of the amino sugar, but not substantially different from the rate of degradation of doxorubicin at pH values between 4.0 and 6.5 suggested that the mechanism of degradation at pH values less than 4 was different to that at pH greater than 4. This hypothesis was reinforced by recently published data from Beijnen et al [184] who showed a difference in the mechanism of degradation of daunorubicin and doxorubicin at pH values above and below pH 4. These authors observed that at pH values less than 4 acidic hydrolysis of doxorubicin, daunorubicin and epirubicin yielded doxorubicinone, daunorubicinone and doxorubicinone respectively and their corresponding amino sugars. At pH values greater than 4 daunorubicin degraded to produce 7,8-dehydro-9,10-desacetyldaunorubicinone (DrVIII) and 7,8,9-bis-anhydro-daunorubicinone (DrVI) (Figure 4.14). At pH values greater than 4 the degradation pattern of doxorubicin has not been elucidated completely although Beijnen et al [184] showed formation of DVIII and minor quantities of other fluorescing compounds. It is probable, therefore, that a similar mechanistic change occurred for epirubicin and that this

change may be linked to the observed changes in the stability of this analogue in acidic solution.

In strongly alkaline solution doxorubicin and epirubicin appeared to degrade at almost the same rate whereas the rate of degradation of daunorubicin was substantially slower. As indicated earlier in this chapter daunorubicin was also more stable in sodium chloride 0.9%, pH 6.47, than either doxorubicin or epirubicin. The data suggest that structural differences in the amino sugar moiety did not affect the rate of degradation in buffered solutions or in infusion fluids. These data are in agreement with data published by Beijnen et al [176,185]. The only structural difference between doxorubicin and daunorubicin is the presence of a hydroxyl group on C14 in the doxorubicin molecule. Beijnen et al postulated that this structural difference must hold the key to the differences in stability observed for these two analogues in alkaline solution but no explanation for the increased stability of daunorubicin in either alkaline buffers or the more alkaline infusion fluids was put forward by these authors.

The increased rate of degradation of doxorubicin and epirubicin may be explained by the fact that the C-9 side chain in these analogues undergoes enolisation in weakly alkaline solution as described in section 4.3.ii. Enolisation results in anion production and may therefore lead to an increase in the rate of oxidation of these two analogues compared to daunorubicin. Enolisation also promotes formation of 7,8-dehydro-9,10-desacetyldaunorubicinone rather than the bis-anhydro derivatives that are

also formed during the degradation of daunorubicin in alkaline solution. In summary, these data suggest that the mechanism of degradation of doxorubicin and epirubicin differs from the mechanism of degradation of daunorubicin in alkaline solution.

A comparison of the rate constants for degradation of each drug in half strength Britton-Robinson buffer, pH 4.35, 5.33 and 6.37, at 25°C, with those for degradation of identical solutions in dextrose 5%, pH 4.36, sodium chloride 0.9%, pH 5.2, and sodium chloride 0.9%, pH 6.47, showed that the rate of degradation in aqueous buffered solutions was much more rapid than in infusion fluids of similar pH values (Tables 4.1, 4.5, 5.3, 5.4 and 5.5 respectively). These and other experimental data showed that at constant temperature, pH and ionic strength the rate of degradation of these drugs was increased by the presence of buffers. Buffer catalysis was also shown in recent experiments by Beijnen et al. These authors observed a non-linear buffer catalysis of daunorubicin between pH 3 and pH 11.5 [185] and doxorubicin at pH values less than or equal to pH 9.5 [184] in acetate, phosphate and carbonate buffers.

Photodegradation, degradation in buffer solutions and drug loss in glass and polypropylene syringes at concentrations of approximately 2mg/ml followed first order kinetics. At low concentrations (approximately 100µg/ml) data plotted as a first order model for solutions stored in glass and PVC containers, showed a biphasic degradation pattern. The initial slope was considered to be due to

adsorptive losses onto the container as the drug loss increased (as a proportion of the total amount) as the concentration of the solution decreased.

Drug loss due to adsorption is expected to be complete within a few hours of the drug coming into contact with the container as theoretically this phenomenon ceases upon formation of a monolayer of the adsorbed drug on the plastic. However, in this experiment "adsorption" appeared to continue for up to eight days. These data suggested that a rapid initial adsorption probably occurred, which played a minor role in the overall loss of drug, followed by a slower dissolution and migration of the drugs into the plastic matrix. This type of model has been suggested for the interaction of glyceryl trinitrate, warfarin and some of the benzodiazepines with PVC [195,196,197]. As the interaction of these drugs with PVC appeared to involve more than one process the term "sorption" was used to denote binding of these drugs to the container.

The amount of drug sorbed to the container was dependent on the properties of the drug, the vehicle, the surface area to volume ratio of the filled container, the time of exposure and the temperature at which the container was stored. Other authors have also reported that sorption depends on the materials from which the container is made [198].

The pH of the vehicle was also shown to alter the amount of drug sorbed as it is the unionised species of the drug, which is more lipophilic than the ionised species, that is expected to be preferentially sorbed to the plastic.

The relative amount of the unionised species is controlled by the pKa of the drug and the pH of the solution. The pKa values for doxorubicin, daunorubicin and epirubicin are 8.4, 8.3 and 8.0 respectively (Table 1.3) therefore as the pH of the solvent increases the amount of drug sorbed is expected to increase. Results in this study clearly showed this relationship as the amount of the parent drug sorbed was greater from sodium chloride 0.9%, pH 6.47, than from sodium chloride 0.9%, pH 5.2, and lowest from dextrose 5%, pH 4.36.

The extent of sorption was also shown to be directly proportional to the surface area of PVC in contact with the solution and decreased as the storage temperature decreased. Drug loss due to sorption in solutions which were frozen and stored at -20^{o}C probably occurred during the time taken for the solution to freeze after preparation and subsequently when the drug was thawed and re-frozen after sampling.

The logarithm of the partition coefficients, log K values, for doxorubicin, epirubicin and daunorubicin between the PVC and the solution were calculated using an equation from Illum and Bundgaard [195] (section 5.3.i) as 0.1, 0.2 and -0.2 respectively. The values obtained by these authors for diazepam, warfarin and glyceryl trinitrate (which were reported to be strongly sorbed to PVC) were 1.2, 1.9 and 1.6 respectively for a similar weight of plastic. These data indicate that in the 8 days required for the studied anthracyclines to equilibrate with the PVC drug loss due to sorption was minimal. The results in Table 5.2 substantiate this statement.

Illum and Bundgaard [195] also reported that the hexane:water partition coefficient was the most useful parameter for the prediction of the sorptive behaviour of a particular drug to PVC. Data in this experiment showed that the butanol:water partition coefficients were also a useful predictor of sorptive behaviour of the studied anthracyclines to PVC.

Chromatograms from admixtures which were stored for about 6 weeks in PVC minibags showed very little evidence of degradation products despite the fact that significant drug loss (greater than or equal to 10%) occurred in some of the admixtures tested. After the minibags were emptied a dull pink coating was observed on the inside surface. This coating was probably due to sorption of parent compound and/or degradation products to the PVC. Recently Beijnen et al [185] reported that aglycone degradation products such as DrVIII, and the bis-anhydro derivatives were adsorbed onto silanised glass. It is possible that these products, as well as the parent drugs, were also sorbed to the inside of the PVC containers used in this experiment.

Eleven repeated freezings and thawings (at ambient temperature) of solutions stored in PVC minibags for 4 weeks, did not result in a significantly greater loss of drug when compared to control solutions which were frozen and re-thawed only twice over a similar period ($p > 0.05$) (Table 5.9). Hoffman et al [177] made similar observations for solutions of doxorubicin frozen and re-thawed six times at ambient temperature.

Solutions stored at -20^{o}C in PVC minibags take four to

five hours to re-equilibrate to room temperature if not artificially heated whereas a single 100ml minibag (frozen at -20 °C) takes about 2 minutes to thaw in a microwave. Uneven distribution of energy may lead to overheating of the solutions and subsequent degradation. For this reason, solutions of these drugs which are frozen in PVC minibags should preferably be thawed at ambient temperature unless thawing in a microwave is strictly controlled. As stated by Ausman et al [200] there are several levels of microwave energy emitted by commercial and domestic appliances which can produce different magnitudes of heating for the same exposure time. Many are not designed to distribute energy evenly and local "hot spots" can develop which can result in overheating of the solution and subsequent degradation.

Statistical analysis of solutions of doxorubicin, daunorubicin and epirubicin stored in PVC bags showed that the storage temperature significantly affected the percentage remaining at the end of the study ($p < 0.05$). As a more detailed analysis (Student's t test) showed that there was no significant difference between the percentage remaining in solutions which were refrigerated and those which were frozen at -20°C ($p > 0.05$), storage at 4°C may be more convenient.

Chromatograms from solutions which were stored in glass tubes in the dark showed a decrease in the parent compound with time. As no degradation products were evident on these traces drug loss was probably due to adsorption onto the container. In the laboratory, when these drugs are used in

low concentrations (less than 100µg/ml), drug loss due to adsorption (as a proportion of the total amount) may become significant. In this situation adsorption may be minimised by storage of such solutions in polypropylene containers.

The data obtained from stability studies for these drugs appear to suggest that doxorubicin and epirubicin (100µg/ml) may be safely stored in dextrose 5% and sodium chloride 0.9% in PVC minibags, for at least 6 weeks, providing that the pH of the sodium chloride is at the lower end of the pharmacopoeial limits (the pH should be within the range pH 5.0 to 7.0). Prolonged storage of these two analogues in infusion fluids at the upper end of this pH range is not recommended unless the solutions are frozen and stored at -20°C. Conversely daunorubicin was chemically stable in either dextrose, pH 4.36, or sodium chloride-containing infusion fluids, between pH 5.0 and 7.0.

At concentrations of approximately 2mg/ml (in Water For Injections) doxorubicin, daunorubicin and epirubicin appeared to be chemically and physically stable (less than 10% degradation) for at least 2 weeks in glass containers and at least 6 weeks in polypropylene syringes at 4°C. This is probably explained by the fact that at concentrations greater than or equal to 500µg/ml drug loss due to adsorption (as a proportion of the total amount) was negligible. These data suggest that at concentrations greater than or equal to 500µg/ml these drugs are also likely to be stable when stored in PVC minibags for similar periods. The differences in stability of these drugs in infusion fluids at different pH values is probably

negligible, in the practical situation, such that these drugs may safely be added to either dextrose or sodium chloride containing infusion fluids.

As there is no preservative included in the formulation, the manufacturers recommend that reconstituted solutions should be discarded after 24 hours if stored at room temperature and 48 hours if stored in the refrigerator. If the drugs are reconstituted and diluted under strict aseptic conditions, such that the risk of microbial contamination is negligible, then the expiry of such solutions stored at $4^{o}C$, may be extended at the discretion of the pharmacist in charge of the reconstitution service.

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APPENDICES

Appendix I

Constituents of Britton-Robinson buffer.

pH	Composition (g/l)				Ionic Strength (M)	KCl added per litre of buffer to produce strength of		
	NaOH	CH_3CO_2H	H_3PO_4	H_3BO_3		0.1M	0.5M	1M
1.81	0.000	2.402	3.920	2.473	0.0134	6.389	36.211	73.489
1.98	0.381	2.288	3.733	2.355	0.0180	6.113	35.935	73.213
3.29	1.333	2.002	3.267	2.061	0.0331	4.987	34.809	72.087
4.35	1.725	1.884	3.075	1.940	0.0417	4.346	34.168	71.446
5.33	2.182	1.747	2.851	1.799	0.0539	3.436	33.258	70.536
6.37	2.483	1.657	2.703	1.706	0.0636	2.713	32.535	69.813
7.96	3.000	1.501	2.450	1.546	0.0952	0.357	30.179	67.457
9.91	3.493	1.353	2.208	1.393	0.1100	--	29.076	66.354
11.98	4.000	1.201	1.960	1.237	0.1280	--	27.734	65.012

Since pH values depend on ionic strength the actual pH of each solution was checked experimentally. The values reported in the Table above are the Britton-Robinson original ones (without potassium chloride).

Potassium chloride was added to produce an ionic strength of 0.5M.

Appendix II

Constituents of Sorenson buffer.

Stock solutions;

A = Disodium citrate 0.1M (21.01g $C_6H_8O_7.1\ H_2O$ + 200ml NaOH 1M per litre)

B = Hydrochloric acid 0.1M

Composition of the buffer;

Xml A + (100 - X) ml B

From Documenta Geigy Scientific Tables

pH 1.2	9ml A	+ (100 - 9) B
pH 2.0	30ml A	+ (100 - 30) B
pH 3.0	39.9 A	+ (100 - 39.9) B
pH 4.0	55.1 A	+ (100 - 55.1) B
pH 5.0	100 A	+ (100 - 100) B

Appendix III

Constituents of Tris buffer.

Stock solutions;

A = Tris 0.2M (24.23g tris [hydroxymethyl] amino-methane/l

B = Hydrochloric acid 0.1M

Composition of the buffer;

25ml A + Xml B made up to 100ml

From Documenta Geigy Scientific Tables;

pH 7.2 25ml A + 44.7ml B made up to 100ml

pH 8.0 25ml A + 27.9ml B made up to 100ml

pH 9.0 25ml A + 5.3ml B made up to 100ml