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Doxorubicin decomposition on storage. Effect of pH, type of buffer and liposome encapsulation

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Summary

An HPLC technique was used to monitor the stability of doxorubicin-HCl (DXR) in aqueous media. In this study pH (4.0 and 7.4), composition of the aqueous medium (Tris or phosphate buffer, cell culture medium), temperature (4–91°C), DXR concentration (range 50–500 μg/ml) and liposome encapsulation of DXR were variables under investigation. The results obtained in this study indicated that pH 4 and 4°C provide optimum shelf-life conditions. At pH 7.4 the DXR decomposition rates increased with the DXR concentration. For the 50–100 μg/ml DXR solutions energies of activation were: 58 (Tris) and 73 (phosphate) kJ/mol between 4 and 61°C. Only in one case (pH 7.4, phosphate buffer and incubation temperature 37°C) did liposome encapsulation affect the DXR decomposition kinetics.

Introduction

Doxorubicin (DXR), a cytostatic drug, is active against a wide range of tumors. Recently, reports were published advocating the use of DXR encapsulated in liposomes (Rahman et al., 1980; Forssen and Tökes, 1981, 1983; Olson et al., 1982; Gabizon et al., 1982; Mayhew et al., 1983; Van Hoesel et al., 1984). These authors claim that liposome encapsulation increases the therapeutic index of the drug: the cardiotoxicity decreases, while the cytostatic action is preserved. An acceptable shelf-life is a prerequisite for the successful introduction of DXR liposomes into

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therapy. Today, liposome dispersions are stored as aqueous dispersions. Alternative techniques like freeze-drying are not operational yet (Crommelin and Van Bommel, 1984; Van Bommel and Crommelin, 1984). It is therefore essential to establish the conditions that provide minimal decomposition rates in aqueous media.

Many studies on the stability of DXR on storage have been published, but only a few investigators used techniques that provide a combination of good selectivity and precision like HPLC (Hoffman et al., 1979; Benvenuto et al., 1981; Poochikian et al., 1981; Williamson et al., 1983; Karlsen et al., 1983). Systematic work to gain insight into the mechanism of degradation is rare (Wassermann and Bundgaard, 1983). It is therefore impossible to make exact predictions on DXR decomposition rates in aqueous systems to be used in pharmaceutical practice. Besides, no data on the effect of liposome encapsulation on the stability of DXR are available.

In this study DXR degradation was investigated under various conditions with respect to buffer type, pH, temperature and DXR concentration using an HPLC technique. Two pH levels (pH 4.0 and 7.4) and two buffer systems (Tris and phosphate) were investigated. The stability of 'free' DXR in solution was compared to the stability of liposome-encapsulated DXR at the same pH levels and in the same buffer systems.

Materials and Methods

Reagents

Doxorubicin-HCl and doxorubicinone-HCl were provided by Farmitalia (Milan) and Lab. Roger Bellon (Neuilly sur Seine, Paris) and used without purification.

Phosphatidylcholine from egg yolk type V-E (PC), phosphatidylserine (PS) and cholesterol were supplied by Sigma Chemicals (St. Louis, MO). All other chemicals were of analytical grade.

Preparation and assessment of the quality of the liposomes

DXR was mixed with the phospholipids (PL) and cholesterol (molar ratio PC : PS : chol = 10 : 1 : 4) in a chloroform/methanol mixture (1 : 1 v/v) in a pear-shaped flask. The mixture was rotary-evaporated under reduced pressure at 40–45°C to yield a film. The flask was left under reduced pressure for at least 2 h. Then the hydration medium and glass beads were added. The medium for hydration (pH 4) consisted of 0.8% sodium chloride and 0.01 mol/l Tris-(hydroxymethyl) aminomethane hydrochloride (Tris) in water. Nitrogen was passed through the buffer for at least 15 min. The film was hydrated under shaking at about 45°C. After complete dispersion, the flask with its contents was placed in a refrigerator. At this stage of preparation 1 ml of the dispersion contained about 30 mg PL and 2.5 mg DXR. Sizing of the liposomes was done by extruding the liposomes sequentially through membrane filters with pore diameters of 0.6 and 0.2 μm (Uni-pore, Bio-Rad, Richmond, CA) under (nitrogen) pressures up to 0.8 MPa. Free DXR was removed by dialysis at 4°C against a 0.01 mol/l Tris/0.8% sodium chloride solution (pH 4). Less than 10% of the DXR was in the 'free' form after dialysis. After

removing the non-liposome-associated DXR, the dispersions contained about 1 mg/ml DXR. If necessary, the conditions selected for investigation (pH, buffer type) were obtained by a relatively short second dialysis step against the selected medium at 4°C.

The mean particle diameter as determined by dynamic light scattering (Nanosizer, Coulter Electronics, Luton, U.K.) was 0.25 μm. The degree of association of DXR with the liposomes (retention) was determined by sampling the dispersion during the incubation (see below) and separating 'free' DXR from liposome bound DXR by gel filtration (Sephadex G50 fine, Pharmacia, Sweden). Then DXR was assayed colorimetrically by reading extinctions at 480 nm. For a number of samples it was established that the HPLC technique (see below) and the colorimetric analysis gave similar results for the determination of the ratio 'free' to bound DXR.

Full details on the experimental techniques used for liposome preparation and characterization have already been published (Crommelin et al., 1983; Crommelin and Van Bloois, 1983).

Instrumentation and analytical procedure

The HPLC-system consisted of a M45 Solvent Delivery System Type 6000A, in combination with a U6K injector (200 μl loop), a prepacked Phenyl-μ-Bondapack (3.9 mm × 30 cm) 10 μm particles column, and a Model 440 UV absorbance detector equipped with a 436 nm filter (all from Waters Associates, Milford, MA). Sample volumes of 100 μl (50 μg/ml DXR) or 10 μl (500 μg/ml DXR) were diluted with 1.00 ml methanol and vortexed for 10 s; 10 μl of the diluted solution was injected. The mobile phase consisted of 32% v/v acetone, 5% v/v sodium dihydrogen phosphate/phosphoric acid buffer (120 mmol/l, pH 3.8), 63% v/v water and 0.3% m/v tetrabutylammonium hydrogen sulphate. A flow rate of 1.0 ml/min was used. The eluent components were filtered through Millipore membrane filters (0.2 μm pore size). The eluent was degassed by ultrasonication prior to use. The syringes for injection were silanized by washing the syringe with a solution of dimethyldichlorosilane (3%) in toluene. The excess of the reagent was removed by washing extensively with methanol. Chromatography was performed at room temperature.

In a number of experiments the DXR concentrations were determined in a similar HPLC configuration as described above, but now equipped with an automatic sampling device that allowed sampling immediately followed by DXR analysis at preset time intervals.

The retention times for DXR and doxorubicinone were 6.6 and 15.1 minutes.

Quantitation of DXR concentrations

Calibration curves in the range of 3–150 ng DXR (absolute injected amounts) were constructed using peak heights. A series of DXR solutions was prepared by diluting 5 μl portions of a stock solution containing 1.2 mg/ml DXR in methanol; 5, 10 or 25 μl of these solutions were injected. Straight calibration lines were obtained (correlation coefficient $r > 0.999$). Intercepts with the ordinate did not differ significantly from zero ($P = 0.95$). At the start and at the end of each incubation experiment reference solutions of DXR and doxorubicinone were run.

Unless otherwise stated the concentrations at t = 0 were taken as 100%. The DXR levels in subsequent samples were calculated as percentages of this first sample. The natural logarithm of the fraction of DXR left in the incubation medium was calculated and graphically plotted against time. Rate constants were calculated if (pseudo) first-order kinetics applied.

Incubation media

The following media were used.

(1) Tris buffers with pH 7.4 or 4.0: 0.01 mol/l Tris and 0.8% sodium chloride. The pH was adjusted with 2 mol/l hydrochloric acid.

(2) Phosphate buffers with pH 4.0 or 7.4: 0.01 mol/l sodium dihydrogen phosphate and 0.8% sodium chloride. The pH was adjusted with 2 mol/l hydrochloric acid or 2 mol/l sodium hydroxide.

(3) A medium used for cell culture work as described by Williams and Gunn (1974). To this medium was added: 10% fetal calf serum, 10^5 I.U. penicillin/l, 0.1 mg streptomycin sulphate/l and 0.25 mg amphotericin/l.

The liposome dispersions were diluted with either the Tris or the phosphate buffers or the cell culture medium to obtain the required DXR concentration. DXR solutions were prepared in a similar way. If necessary, the Tris/sodium chloride medium of the liposome dispersion was replaced by the desired medium by dialysis.

Incubation

Incubations at 4°C. One ml samples of DXR-containing solutions or liposome dispersions (100 μg/ml) were incubated in various buffers in 10 ml glass vials in a refrigerator. The solutions were deoxygenated by passing through nitrogen before dissolving DXR. In some cases polypropylene tubes were used, too, for comparison.

During storage, data on the DXR content were collected in triplicate experiments. The DXR concentration of each sample vial was determined in duplicate.

The following buffers were used: Tris pH 4.0 and 7.4 as well as phosphate pH 4.0 and 7.4.

Incubations at 37°C. Two sets of experiments were carried out.

(a) Solutions of 50 μg/ml were incubated in a thermostatted teflon container that was connected to the automatic sampling HPLC system. The following buffer systems were used: Tris pH 4.0 and 7.4 as well as phosphate pH 4.0 and 7.4. No liposome dispersions were tested at this concentration.

(b) Solutions or dispersions containing 500 μg/ml DXR instead of 50 μg/ml were incubated. Here the following conditions were applied: buffer systems containing Tris or phosphate at pH 4.0 and 7.4 as well as the cell culture medium (pH 7.4). All samples containing 500 μg/ml were incubated in the teflon container mentioned before.

Incubations at 61°C. These incubations were performed as mentioned under 'Incubations at 37°C'. The 500 μg/ml DXR samples were studied only in Tris and phosphate buffers at pH 7.4. No liposome dispersions were tested.

Incubations at 72°C. These incubations were performed as mentioned under

'Incubations at 37°C (a)'.Only the Tris and phosphate buffers at pH 7.4 were tested.

Incubations at 91°C. These incubations were performed as mentioned under 'Incubations at 37°C (a)'. Only Tris and phosphate buffers at pH 4.0 were tested.

As a rule the pH was determined at the start and at the end of the experiment. Upon incubation a tendency to a pH increase was observed for all buffer systems. The results were discarded when the pH changed more than 0.3 (for pH 7.4) or 1.0 (for pH 4.0) during the incubation.

Results and Discussion

Storage of DXR (100 μg/ml DXR) in solution or encapsulated in liposomes at 4°C

The DXR concentration was determined as a function of time in aqueous solutions and liposome dispersions. In both buffer systems no significant DXR degradation was recorded over a 60-day period of storage at pH 4.0. More than 80% of the DXR in the liposome dispersions remained liposome-associated over the period of investigation. Storage of the DXR solutions in Tris buffer in polypropylene tubes or glass vials at pH 4.0 gave similar results. At pH 7.4 a substantial decomposition of DXR occurred. In the time frame studied (42 days) (pseudo) first-order kinetics described the degradation profiles in a satisfactory way. Rate constants and the corresponding half-lives are presented in Table 1. These data are only indicative as the calculated half-lives surpassed the actual storage time.

Under the chosen conditions the DXR decomposition rate in the phosphate buffer (pH 7.4) solution was lower than in the Tris buffer, pH 7.4. Encapsulation of DXR in liposomes in this phosphate buffer, pH 7.4, tended to accelerate the decomposition rate. Liposome encapsulation did not affect the DXR degradation rate in the Tris buffer. In the latter case a possible—slight—difference in DXR decomposition rate might be masked, as the retention of DXR dropped to 60% during the first days of incubation. After this initial loss DXR leakage continued at a much lower rate.

TABLE 1

DXR-DEGRADATION ON STORAGE AT 4°C FOR pH 7.4 BUFFERS. FIRST-ORDER RATE CONSTANT k (h^{-1}) AND HALF-LIFE $t_{1/2}$ (DAYS)

Buffer	DXR		DXR-liposomes	
	k	$t_{1/2}$	k	$t_{1/2}$
Tris	94×10^{-4}	74	96×10^{-4}	72
phosphate	71×10^{-4}	98	114×10^{-4}	61

DXR concentration (about 100 μg/ml) as determined after 7 days was taken as 100%. Degradation was monitored over 42 days. DXR contents of 3 vials were measured in duplicate weekly. DXR associated with the liposomes was 60% after 7 days. A slow decrease down to 40% liposome-associated DXR occurred on prolonged storage (42 days).

TABLE 2

FIRST-ORDER RATE CONSTANT k (h^{-1}) AND HALF-LIFE $t_{1/2}$ (h) FOR DXR DECOMPOSITION UNDER VARIOUS CONDITIONS FOR pH, BUFFER AND TEMPERATURE

Buffer, pH	Temperature (°C)							
	37		61		72		91	
	k	$t_{1/2}$	k	$t_{1/2}$	k	$t_{1/2}$	k	$t_{1/2}$
Tris, pH 4.0	*		*		n.d.		46×10^{-3}	15
Tris, pH 7.4	7×10^{-3}	99	22×10^{-3}	32	115×10^{-3}	6	n.d.	
phosphate, pH 4.0	*		*		n.d.		58×10^{-2}	12
phosphate, pH 7.4	14×10^{-3}	50	62×10^{-3}	11	420×10^{-3}	1.7	n.d.	

* = less than 10% degradation over a 40-h period of incubation. n.d. = not determined. Concentration of DXR at t = 0: 50 μg/ml.

Temperature dependence of DXR decomposition (50 μg/ml DXR)

DXR solutions (50 μg/ml DXR) were incubated at various temperatures. The temperature range was selected to provide appropriate data for an Arrhenius

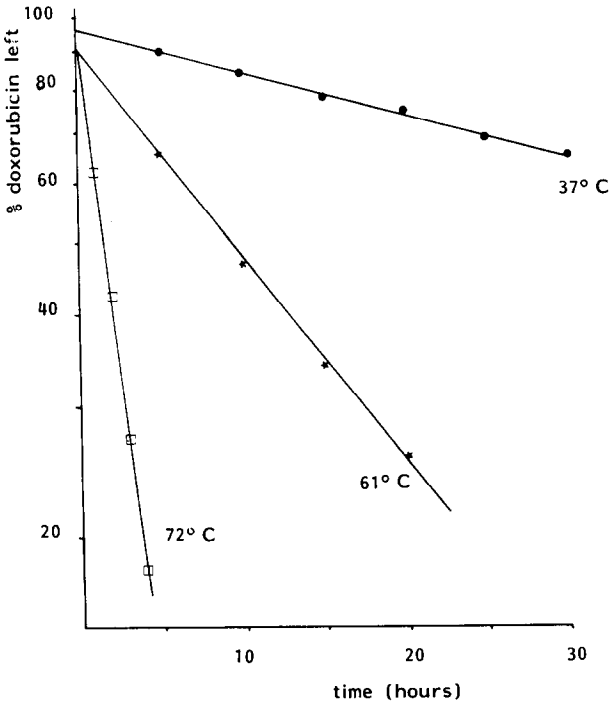

Fig. 1. Typical example of the temperature influence on the decomposition rate of DXR (50 μg/ml). Phosphate buffer; pH 7.4. DXR concentration at t = 0 is taken as 100%. Lines are *calculated* according to least-squares regression analysis.

analysis of the degradation process. A typical example of a family of curves is presented in Fig. 1 for the phosphate buffer system at pH 7.4.

This figure shows that a (pseudo) first-order kinetic model describes the degradation profiles well. Rate constants for the phosphate buffer tended to exceed the Tris buffer rate constants under these conditions (see Table 2).

Arrhenius plots were constructed for the Tris and phosphate buffer systems at pH 7.4 to gain an insight into the order of magnitude of the thermodynamic parameters that control the DXR degradation process. DXR solutions with concentrations in the 50–100 μg/ml range were used. The plots are shown in Fig. 2. For the data points at 4, 37 and 61°C least-squares regression analyses resulted in slopes of $-3.0 \times 10^{+3}$ (correlation coefficient $r = 0.994$) and $-3.8 \times 10^{+3}$ ($r = 0.993$) for the Tris and phosphate solutions, respectively. From these slopes the corresponding energies of activation were calculated: 58 (Tris, pH 7.4) and 73 (phosphate, pH 7.4) kJ/mol. For both the Tris and phosphate buffers the degradation rate constants that were determined at 72°C deviate from the values predicted by extrapolation of the

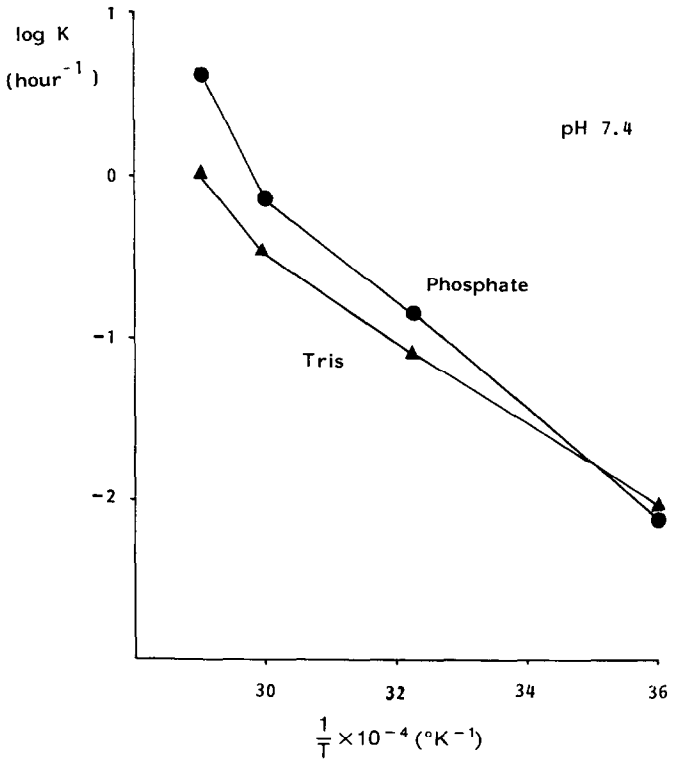

Fig. 2. Arrhenius plots of DXR decomposition rate constants in the phosphate and Tris buffer systems at pH 7.4. DXR concentrations were in the range from 50 to 100 μg/ml DXR. T = absolute temperature (°K)

calculated lines. A change in the main rate-controlling decomposition pathway in the temperature range between 61 and 72°C may offer an explanation.

Concentration dependence of DXR decomposition

The concentration of DXR was found to be a critical parameter for its stability. In Table 3 DXR decomposition rate constants are shown for 50 μg/ml and 500 μg/ml DXR solutions in the Tris and phosphate buffer systems for pH 7.4 at 37 and 61°C.

Here for both degradation processes (pseudo) first-order kinetics describe the decomposition profile well. It is remarkable that at both temperature levels DXR was decomposed faster in the more concentrated solutions. Tavoloni et al. (1980) concluded on the basis of fluorescence data that the decomposition rates of DXR solutions (protected from light) were not concentration dependent in the range from 10 to 500 μg/ml DXR. The discrepancy between the data presented here and theirs might be ascribed to the poor specificity of the fluorescence technique compared to the HPLC analysis presented here. Poochikian et al. (1981) also reported that the decomposition of DXR was concentration independent. Unfortunately, they did not present any experimental data to support their statement.

Dependence of DXR degradation on liposome encapsulation and the composition of the medium

At 37°C the degradation profiles for DXR in different liposome dispersions and 'free' in solutions were compared (Table 4). With the exception of the phosphate pH 7.4 buffer, liposome encapsulation did not affect the decomposition kinetics significantly. A pronounced influence of the composition of the incubation medium was observed. At this temperature the decomposition rate increased in the following order (cf. Table 1): Tris < phosphate < cell culture medium. The reason for the dramatic increase in decomposition rate when working with the culture medium has to be established yet.

TABLE 3

CONCENTRATION DEPENDENCE OF DXR DECOMPOSITION. FIRST-ORDER RATE CONSTANT k (h^{-1}) AND HALF-LIFE $t_{1/2}$ (h) FOR 2 TEMPERATURES AND 2 BUFFERS

Buffer, pH	Temperature (°C)							
	37 DXR concentration (μg/ml)				61 DXR concentration (μg/ml)			
	50		500		50		500	
	k	$t_{1/2}$	k	$t_{1/2}$	k	$t_{1/2}$	k	$t_{1/2}$
Tris, pH 7.4	7×10^{-3}	99	16×10^{-3}	42	22×10^{-3}	32	86×10^{-3}	8
phosphate, pH 7.4	14×10^{-3}	50	64×10^{-3}	11	62×10^{-3}	11	186×10^{-3}	4

TABLE 4

FIRST-ORDER DEGRADATION RATE CONSTANT k (h^{-1}) AND HALF-LIFE $t_{1/2}$ (h) FOR DXR DECOMPOSITION. A COMPARISON BETWEEN DEGRADATION KINETICS OF 'FREE' AND LIPOSOME-ENCAPSULATED DXR AND THE INFLUENCE OF THE BUFFER COMPOSITION ON THE DEGRADATION RATE

Buffer, pH	'free' DXR		liposome-associated DXR	
	k	$t_{1/2}$	k	$t_{1/2}$
Tris, pH 4	*	*	*	*
Tris, pH 7.4	16×10^{-3}	43	17×10^{-3}	41 [a]
phosphate, pH 4	*	*	*	*
phosphate, pH 7.4	64×10^{-3}	11	29×10^{-3}	24 [b]
cell culture medium	146×10^{-3}	5	137×10^{-3}	5 [c]

DXR concentration = 500 μg/ml. Incubation temperature: 37°C.
(a) DXR retention after incubating 2 h 70%, 4 h 62%, 6 h 60%.
(b) DXR retention after incubating: 2 h 76%, 4 h 75%, 6 h 75%.
(c) DXR retention after incubating: 2 h 65%, 4 h 55%, 6 h 52%.
* = less than 10% degradation over a 7-h period of incubation.

Degradation products

Although no attempts were made to investigate the degradation pathway of DXR in detail, a few observations are worth mentioning. A compound with a retention time equal to doxorubicinone was detected at 37°C and higher temperatures when incubating at pH 4.0. In the first stage of incubation, mass balance calculations indicated a quantitative conversion of DXR to doxorubicinone. Later on, other components were formed as well.

At pH 7.4 no doxorubicinone was detected. Unidentified compounds with retention times of 6.3 and 7.6 min were recorded.

Conclusions

At pH 4.0 no significant decomposition was observed; this holds for DXR solutions and liposome dispersions (in Tris as well as in phosphate buffer) that were stored for at least 40 days protected from light at 4°C. At pH 4.0 the buffer capacity of the Tris buffer is low. However, this system provides physicochemically well-defined and stable DXR-containing liposomes (Crommelin et al., 1983; Crommelin and Van Bloois, 1983). These dispersions were successfully tested in vivo (Van Hoesel et al., 1984).

At pH 7.4 the DXR decomposition rates are larger than at pH 4.0. (Pseudo) first-order kinetics apply. Only in the phosphate buffer (pH 7.4) DXR encapsulation in liposomes affected the decomposition kinetics considerably. It was reported before that DXR strongly interacts with these negatively charged bilayers (Crommelin et al., 1983; Crommelin and Van Bloois, 1983). The results in the present study indicate that in the Tris buffer the part of the molecule being sensitive to degradation is freely available to the aqueous medium. Under the conditions studied a pH of

4.0 is the pH to be preferred for an optimum shelf-life. At pH 7.4 the composition of the medium has a significant influence on DXR stability. The decomposition rate constants increased in the following order: Tris < phosphate < cell culture medium. In this cell culture medium, which is used for DXR cytotoxicity testing, 50% of the DXR degraded in 5 h at 37°C. This raises the question: what is the rationale of incubating DXR-solutions with (tumor) cells for longer periods of time at 37°C in media with a comparable composition? A number of degradation products is formed. This causes an extremely complex situation. It is difficult to discriminate between the cytotoxic action of the parent compound and the degradation products. In our laboratory work is in progress to address the problem related to the chemical stability of DXR in a systematic way.

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