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Chemical stability of *N*-trifluoroacetyldoxorubicin-14-valerate (AD-32) in aqueous media and after liposome encapsulation

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Summary

The chemical stability of *N*-trifluoroacetyldoxorubicin-14-valerate (AD-32) in aqueous media has been studied using a stability-indicating high performance liquid chromatographic technique. In this study the influences of pH, ionic strength, the presence of cosolvents, i.e. acetonitrile, methanol, temperature and liposome encapsulation on the degradation kinetics of AD-32 were investigated. AD-32 can be solubilized using a cosolvent while on increasing cosolvent concentration stabilization occurs. The compound shows maximum stability at pH values around 4, whereas after liposome encapsulation during a period of 13 weeks no significant degradation of AD-32 was observed. In addition, the identities of the degradation products of AD-32 in acidic as well as alkaline media are presented.

Introduction

N-trifluoroacetyldoxorubicin-14-valerate (AD-32) is a recently developed disubstituted derivative of the anthracycline antibiotic doxorubicin (Fig. 1) (Israel et al., 1975; Crooke, 1981). Chemically, AD-32 differs from doxorubicin in the presence of a trifluoroacetylgroup attached to the 3′-amino-group and in the presence of the valerate moiety at C14. It will be clear that the physico-chemical and biological characteristics of AD-32 are completely different from those of doxorubicin.

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It is observed that AD-32 is therapeutically more active and less toxic than doxorubicin in experimental tumor systems and in animals (Vecchi et al., 1978; Henderson et al., 1978; Niell et al., 1986). The mechanism of antineoplastic action of AD-32 is different from the intercalative DNA-binding mechanism of doxorubicin. This is demonstrated by the observation that AD-32 does not bind to double-stranded DNA (Israel et al., 1980; Arcamone, 1981). Until now only a few in vivo biotransformation studies give some information about the stability of AD-32 (Israel et al., 1978; Barbieri et al., 1979).

This article describes a systematic study concerning the degradation of AD-32 in aqueous/organic solutions. The aim was to obtain more

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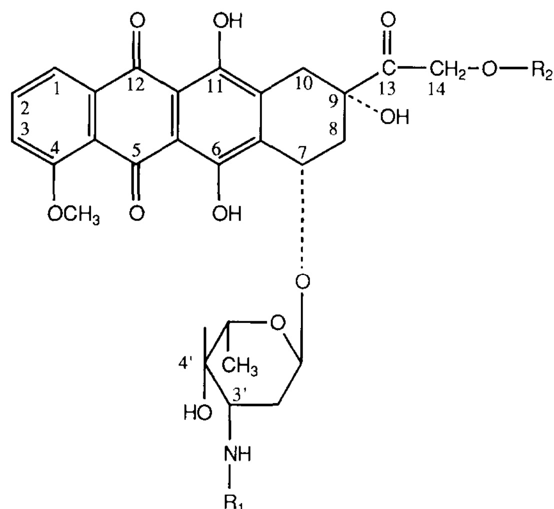

Fig. 1. Structures of the anthracyclines doxorubicin, AD-32 and AD-41.

	R_1	R_2
Doxorubicin	H	H
AD-32	$COCF_3$	COC_4H_9
AD-41	$COCF_3$	H

detailed insight in the nature of the AD-32 degradation. The effects of pH, ionic strength, cosolvent and temperature on the degradation as well as the identitites of the degradation products have been studied. Also the chemical stability of AD-32 after liposome encapsulation has been investigated.

Materials and Methods

Chemicals

AD-32 and *N*-trifluoroacetyldoxorubicin (AD-41) were kindly provided by Dr. S. Penco, Farmitalia (Milan, Italy). Doxorubicinone was synthesized as described by Beijnen et al. (1985). Phosphatidylcholine and phosphatidylserine were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All other chemicals used were of analytical grade and deionized water was used throughout.

Degradation solutions

The kinetic studies were performed in water/acetonitrile mixtures (1 : 1, v/v), except in experiments where the type and the percentage of cosolvent was studied as well as in degradation studies with liposome-entrapped AD-32. The aqueous components were 0.01 M buffer solutions. The following buffers were used: pH 0.5–3, perchloric acid; pH 3–5, acetate; pH 5–8, phosphate and pH 8–11, borate. pH Values were measured using a glass reference electrode and pH-meter (Methrom, E516 Triskop, Herisau, Switzerland). To each degradation solution sodium edetate was added to a concentration of 5×10^{-4} M. A constant ionic strength ($\mu = 0.3$) in the aqueous solutions was maintained by addition of appropriate amounts of sodium chloride, except for the solutions where the hydrogen ion concentration exceeded 0.3 M and for the solutions used in the experiments where the ionic strength influence was investigated. The influences of ionic strength, type and percentage of cosolvent and temperature on the degradation rate have been studied at pH 0.9 and at pH 8.5.

Encapsulation of AD-32 in liposomes

The AD-32 containing liposomes were prepared according to the procedure described for doxorubicin by Janssen et al. (1985) with a few modifications. AD-32 was mixed with phosphatidylcholine/phosphatidylserine (10 : 1 molar) in chloroform and evaporated to dryness, for at least 2 h, on a rotary vaporizer under reduced pressure to yield a film. Subsequently the hydration medium (0.8% sodium chloride and 10 mM Tris-(hydroxymethyl) aminomethane hydrochloride in water) and glass beads were added.

The film was hydrated under shaking and subsequently placed in a refrigerator for one night. Sizing of the liposomes was done by extruding the liposomes through nucleopore membrane filters with pore sizes of 0.6 μm and 0.2 μm (Unipore, Bio Rad, Richmond, CA) under nitrogen pressure. After extruding no AD-32 crystals were present. The mean particle diameter was determined by dynamic light scattering (Nanosizer, Coulter Electronics, Luton, U.K.) and found to be 339 nm. The liposome dispersion was stored at 4° C and at

certain time intervals samples were analyzed for the content of AD-32.

HPLC system

HPLC analysis was carried out using a model M-6000 pump, a model U6K injector and a Model 440 dual wavelength UV-detector (all from Waters Assoc., Milford, MA, U.S.A.) operating at 254 nm and 436 nm. Peak heights and peak areas of undegraded AD-32 were measured with a SP-4270 integrator (Spectra Physics, San Jose, U.S.A.) and used for quantitation.

The stationary phase was Lichrosorb RP8 (5 μm), prepacked in a 12.5 cm × 4.0 mm (i.d.) column (Merck, Darmstadt, F.R.G.). The eluents consisted of water-acetonitrile (50 + 50, v/v). Before mixing with the organic modifier the pH of the water phase was adjusted to 2.5 with phosphoric acid.

Chromatography was carried out at room temperature. The flow rate of the mobile phase was 1.5 ml/min. Samples of 20 μl were injected onto the column. In the concentration range of interest (2–20 μg/ml) standards of AD-32 were chromatographed. The calibration curves constructed show linear responses ($r > 0.999$).

Kinetic studies

The kinetic studies in aqueous/organic solutions were carried out over at least three half-lives in the dark and the temperature of study was $50 \pm 0.2°$C throughout. The reactions were initiated by adding 40 μl of a solution of AD-32 (1 mg/ml) in dimethylformamide to 2 ml preheated solvent to give an initial concentration of 20 μg/ml. The degradation experiments were carried out in silanized glass flasks. Silanization was executed with dimethylsilane in toluene (3%, v/v) with subsequent washing with methanol. At fixed time intervals samples were withdrawn and directly analyzed with a stability-indicating high performance liquid chromatographic (HPLC) method. The quantity of AD-32 in liposome suspensions was measured after shaking 100 μl liposome dispersion with 10 ml acetonitrile 30 μl of the mixture was injected onto the HPLC-system.

Identification of degradation products

Ultraviolet-visible (UV-VIS) absorbance spectra of the degradation products were recorded by scanning with a PU 4021 Photo Diode Array Detector (Pye Unicam Ltd., Cambridge, U.K.) on line with the HPLC-system. Fluorescence spectra of some of the components were obtained with a Perkin-Elmer Fluorescence Spectrophotometer 204 (Hitachi Ltd., Tokyo, Japan) after chromatographic isolation. Thin layer chromatography (TLC) of chloroform extracts of degradation mixtures was performed on silica gel plates (DC Fertig platten Kieselgel 60, Merck, Darmstadt, F.R.G.). The eluent was chloroform–methanol–acetic acid (93 + 5 + 2, v/v/v) and the migrating distance 15 cm.

Results and Discussion

Analytical methodology

Degradation studies had to be performed in water/acetonitrile or water/methanol mixtures due to the poor water solubility of AD-32 and its degradation products and its tendency to adsorb to all kinds of materials. This problem could be solved by using silanized glassware and water/acetonitrile or water/methanol mixtures.

Traces of metal impurities may catalyze the degradation of AD-32. This effect could be eliminated by the addition of sodium edetate as a chelating agent.

HPLC-analysis

The developed HPLC-assay is stability-indicating. This is illustrated in Fig. 2 and Fig. 3. It is obvious that the degradation products are well resolved from the parent compound. The capacity factors of the components are listed in Table 1.

Degradation products

An overall degradation scheme for AD-32 is given in Fig. 4. The identities of the degradation products were confirmed by comparison of the elution volumes with those of reference compounds, using TLC as well as HPLC with on-line UV-VIS detection with a photodiode array detector or fluorescence detection. In acidic medium

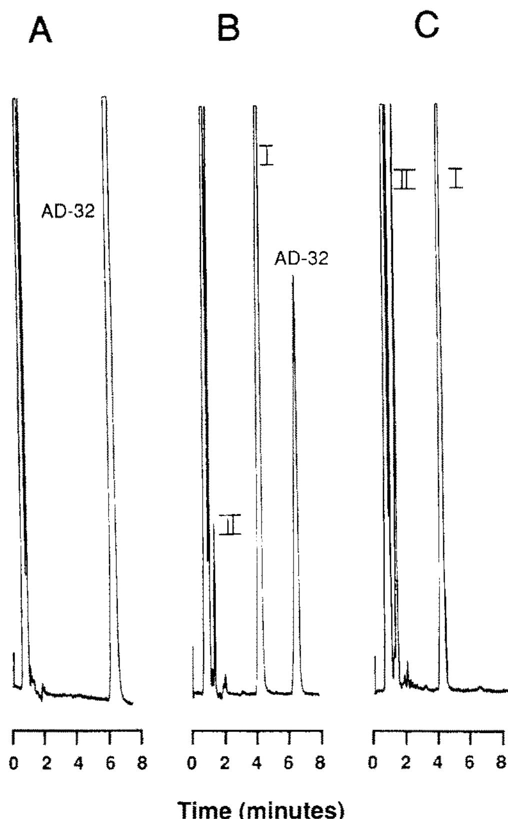

Fig. 2. HPLC chromatograms (for conditions see text) during degradation of AD-32 at pH 0.9; (A) $t = 0$ h; (B) $t = 1$ h; (C) $t = 24$ h; **I**: doxorubicinone-14-valerate; **II**: doxorubicinone.

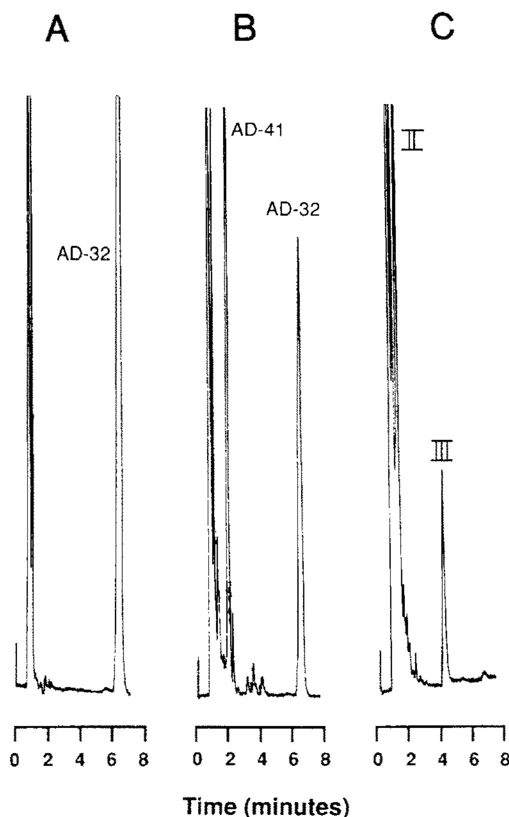

Fig. 3. HPLC chromatograms (for conditions see text) during degradation of AD-32 at pH 8.5; (A) $t = 0$ h; (B) $t = 1$ h; (C) $t = 24$ h; **II**: doxorubicinone; **III**: compound **III**.

glycosidic anthracyclines, such as doxorubicin, undergo a proton-catalyzed cleavage of the glycosidic bond, yielding an aminosugar and a 7-hydroxyaglycone (Beijnen et al., 1985). From this study it appears that the initial degradation step of AD-32 in acidic media is the formation of the corresponding aglycone doxorubicinone-14-valerate. However, doxorubicinone-14-valerate is, in contrast with aglycones of anthracyclines like doxorubicin (Beijnen et al., 1985), not stable in acidic media and degrades further into doxorubicinone (Fig. 4). In alkaline medium the initial degradation step of AD-32 involves hydrolysis of the C14-valerate ester linkage, yielding *N*-trifluoroacetyldoxorubicin (AD-41). AD-41 is the major metabolite of AD-32 found in blood, urine and bile of animals treated with AD-32 (Israel et al., 1978). AD-41 subsequently degrades into doxorubicinone and an aglycone (**III**, Fig. 4) with a fully aromatized A-ring (Beijnen et al., 1987). This conversion is in good agreement with the degradation of doxorubicin in alkaline medium (Beijnen et al., 1986). When AD-41 is treated with acid doxorubicinone is also formed.

TABLE 1

Capacity factors (k′) of some anthracycline compounds

Compound	k'
Doxorubicinone	0.73
Doxorubicin	1.1
AD-41	1.5
Doxorubicinone-14-valerate	4.6
Compound **III**	4.6
AD-32	7.5

For HPLC conditions see text.

Fig. 4. Degradation pattern of AD-32 in acidic as well as alkaline solution.

Degradation kinetics

The degradation of AD-32 in acidic as well as in alkaline media displays pseudo first-order kinetics. This has been studied for over at least 3 half-lives. The presence of acetonitrile or methanol has no influence on this kinetic behaviour. The observed pseudo-first order rate constant (k_{obs}) for the overall degradation rate was calculated by linear regression analysis of a plot of the natural logarithm of the residual concentration of AD-32 versus time:

$$\ln[\text{AD-32}]_t = \ln[\text{AD-32}]_0 - k_{obs} \cdot t \quad (1)$$

where $[\text{AD-32}]_t$ and $[\text{AD-32}]_0$ are the concentrations AD-32 at time t and the initial concentration, respectively.

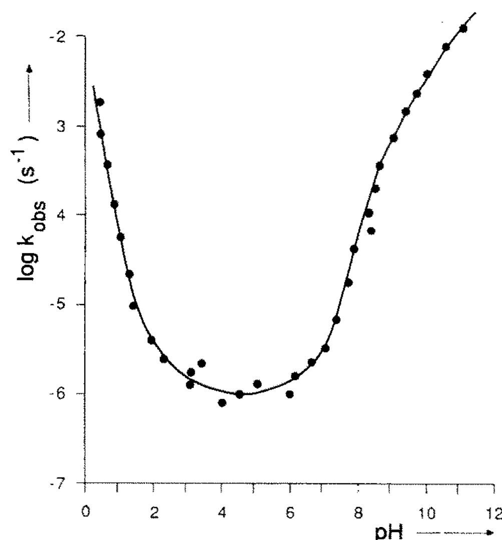

Fig. 5. Log k_{obs}–pH profile for the degradation of AD-32.

Standard deviation in k_{obs}

The standard deviation in k_{obs} was determined at pH 0.9 and at pH 8.5. The mean values of k_{obs} $\pm$S.D. are $4.10 \pm 0.10 \times 10^{-4}$ s^{-1} ($n = 6$) and $2.17 \pm 0.13 \times 10^{-4}$ s^{-1} ($n = 8$). All other k_{obs} values are determined in duplicate.

Influence of pH

The influence of pH of the aqueous component of the solvent has been studied in the range 0.5–11. During these experiments ionic strength, buffer concentration of the aqueous component and percentage acetonitrile were maintained constant. It is obvious that the degradation rate of AD-32 is influenced by the pH of the medium as illustrated by the log k_{obs}–pH profile (Fig. 5). In the range 0.5 < pH < 8 AD-32 consists almost exclusively in the nonprotonated form, in contrast with its parent compound doxorubicin in which the C3′ amino group has a pK_a of 7.5 (Sturgeon and Schulman, 1977; Beijnen et al., 1986). The presence of the trifluoroacetyl group in AD-32 causes the loss of the prototropic properties of the amino group. Accordingly, in the log k_{obs}–pH profile no inflexions due to prototropic equilibria can be observed in the pH range 0.5–8.

In the pH range 8–11 the prototropic equilibrium of the first phenolic hydroxyl group in the aglycone part of the molecule may be involved, indicated by a slight inflexion in the log k_{obs}–pH profile at pH around 9.5, the pK_a of this hydroxyl group in doxorubicin in aqueous solution being 9.5 (Sturgeon and Schulman, 1977; Beijnen et al., 1986). The pH of maximum stability for AD-32 is around 4.

Influence of ionic strength

The influence of the ionic strength was investigated at pH 0.9 and 8.5 by adding various amounts of sodium chloride to the degradation solution, while the buffer concentration and the temperature are kept constant. Within the range investigated ($\mu = 0.1$–0.7) at pH 8.5 no influence of the ionic strength on the degradation rate of AD-32 was observed. However, at pH 0.9 the degradation rate decreased somewhat on increasing the ionic strength of the reaction medium (Table 2).

TABLE 2

Influence of ionic strength (μ) on the degradation of AD-32 at $t = 50\,°C$ and 5×10^{-4} M EDTA

pH	μ	$k_{obs}(s^{-1})$	pH	μ	$k_{obs}(s^{-1})$
0.9	0.12	4.5×10^{-4}	8.5	0.13	2.4×10^{-4}
0.9	0.32	4.1×10^{-4}	8.5	0.33	2.2×10^{-4}
0.9	0.52	3.4×10^{-4}	8.5	0.53	2.6×10^{-4}
0.9	0.72	3.1×10^{-4}	8.5	0.73	2.5×10^{-4}

Usually the degradation of a neutral molecule is independent of the ionic strength (Martin et al., 1983). A possible explanation for this result can be that the higher salt concentration in the vicinity of the glycosidic bond in the AD-32 molecule stabilizes the drug by protecting it against attacking protons in acidic solution.

Influence of percentage and type of cosolvent

The influence of the nature and percentage cosolvent on the degradation of AD-32 has been studied whereby the aqueous solvent component was adjusted to pH 0.9 or pH 8.5. The ionic strength of the aqueous solution and the temperature were kept constant. The results of these experiments are listed in Table 3. From Table 3 it can be concluded that cosolvents have a stabilizing effect on the AD-32 degradation. This may be assigned to the change in dielectrical constant of the medium.

TABLE 3

Influence of nature and percentage of cosolvent on the degradation of AD-32 at $t = 50\,°C$, $\mu = 0.3$ and 5×10^{-4} M EDTA

% (v/v) cosolvent	pH 0.9		pH 8.5	
	$k_{obs}(s^{-1})$ acetonitrile	$k_{obs}(s^{-1})$ methanol	$k_{obs}(s^{-1})$ acetonitrile	$k_{obs}(s^{-1})$ methanol
20	7.2×10^{-4}	n.d.	4.9×10^{-4}	n.d.
25	8.1×10^{-4}	n.d.	4.3×10^{-4}	n.d.
30	6.6×10^{-4}	n.d.	3.8×10^{-4}	n.d.
35	6.2×10^{-4}	5.1×10^{-4}	3.2×10^{-4}	3.1×10^{-4}
40	5.6×10^{-4}	4.4×10^{-4}	3.1×10^{-4}	2.5×10^{-4}
50	4.1×10^{-4}	3.0×10^{-4}	2.2×10^{-4}	2.7×10^{-4}
70	3.6×10^{-4}	1.4×10^{-4}	1.4×10^{-4}	1.3×10^{-4}
90	4.0×10^{-4}	4.6×10^{-5}	6.9×10^{-6}	4.9×10^{-5}

n.d.: not determined, due to adsorption of AD-32 to the glass walls.

TABLE 4

Influence of temperature on the degradation of AD-32 in terms of activation energies and frequency factors ($\mu = 0.3$; 5×10^{-4} M EDTA)

pH	E_a (kJ/mol)	A (s^{-1})
0.9	114	8.3×10^{14}
8.5	85	1.2×10^{10}

Influence of temperature

The effect of temperature on the degradation rate was determined at pH 0.9 and pH 8.5 over the range 25–65°C. The Arrhenius relationship was obeyed (Eqn. 2).

$$\ln k_{obs} = \ln A - (E_a/RT) \quad (2)$$

A represents the frequency factor, E_a the activation energy, R the molar gas constant and T the temperature in K.

By plotting the reciprocal of T versus $\ln k_{obs}$ the activation energy and the frequency factor can be calculated. The results are summarized in Table 4.

Influence of liposome encapsulation

The AD-32 stability in liposome dispersion was monitored over a period of 13 weeks at pH 4 and 4°C. No significant degradation was observed during this period, indicating that AD-32 under these circumstances is stable. Data of the stability of the free compound in water at pH 4 and 4°C, however, are not available, due to the lack in solubility of AD-32 in this medium. No conclusions can therefore be drawn about a possible stabilizing effect of liposome encapsulation of AD-32. During the period of storage the liposomes remained physically stable.

References

Arcamone, F., *Doxorubicin. Antineoplastic antibiotics,* Academic Press, New York, 1981.

Barbieri, B., Abbruzzi, R., Benigni, A., Rizzardini, M., Donelli, M.G., Garattini, S. and Salmona, M., Quantitative thin-layer chromatographic measurement of *N*-trifluoroacetyladriamycin-14-valerate (AD-32) and trifluoroacetyladriamycin (AD-41) in blood and tissues. *J. Chrom. Biomed. Appl.,* 163 (1979) 195–200.

Beijnen, J.H., Wiese, G. and Underberg, W.J.M., Aspects of the chemical stability of doxorubicin and seven other anthracyclines in acidic solution. *Pharm. Weekbl. Sci. Ed.,* 7 (1985) 109–116.

Beijnen, J.H., Van der Houwen, O.A.G.J. and Underberg, W.J.M., Aspects of the degradation kinetics of doxorubicin in aqueous solution. *Int. J. Pharm.,* 32 (1986) 123–131.

Beijnen, J.H., Potman, R.P., van Ooijen, R.D., Driebergen, R.J., Voskuilen, M.C.H., Renema, J. and Underberg, W.J.M., Structure elucidation and characterization of daunorubicin degradation products. *Int. J. Pharm.,* 34 (1987) 247–257.

Crooke, S.T., The anthracyclines. In Crooke, S.T. and Prestayko, A.W. (Eds.), *Cancer and Chemotherapy, Vol. 3, Antineoplastic Agents,* Academic Press, New York, 1981, pp. 111–132.

Henderson, I.C., Billingham, M., Israel, M., Krishan, A. and Frei, E. III., Comparative cardiotoxicity studies with adriamycin (ADR) and AD-32 in rabbits. *Proc. Am. Assoc. Cancer Res.,* 19 (1978) 158.

Israel, M., Modest, E.J. and Frei, E., III, *N*-Trifluoroacetyladriamycin-14-valerate, an analog with greater experimental antitumor activity and less toxicity than adriamycin. *Cancer Res.,* 35 (1975) 1365–1368.

Israel, M., Pegg, W.J. and Wilkinson, P.M., Urinary anthracycline metabolites from mice with adriamycin and *N*-trifluoroacetyladriamycin-14-valerate. *J. Pharmacol. Exp. Ther.,* 204 (1978) 696–701.

Israel, M., Wilkinson, P.M. and Osteen, R.T., Pharmacology studies with adriamycin and *N*-trifluoroacetyladriamycin-14-valerate (AD-32) in *Cynomolgus* monkeys: additional evidence for the absence of an adriamycin–prodrug mechanism for AD-32. In Crooke S.T. and Reich S.D. (Eds.), *Anthracyclines: Current Status and New Developments,* Academic Press, New York, 1980, pp. 431–444.

Janssen, M.J.H., Crommelin, D.J.A., Storm, G. and Hulshoff, A., Doxorubicin decomposition on storage. Effect of pH, type of buffer and liposome encapsulation. *Int. J. Pharm.,* 23 (1985) 1–11.

Martin, A., Swarbrick, J. and Cammarata, A., *Physical Pharmacy,* Lea and Febiger, Philadelphia, 1983, pp. 371–383.

Niell, H.B., Hunter, R.J., Herrod, H.G. and Israel, M., The activity of adriamycin (ADR) and *N*-trifluoroacetyladriamycin-14-valerate (AD-32) against human bladder tumor cell lines. *Am. Ass. Cancer Res.,* 27 (1986) 412.

Sturgeon, R.J. and Schulman, S.G., Electronic absorption spectra and protolytic equilibria of doxorubicin: direct spectrophotometric determination of microconstants. *J. Pharm. Sci.,* 66 (1977) 958–961.

Vecchi, A., Cairo, M., Mantovani, A., Sironi, M. and Spreafico, F., Comparative antineoplastic activity of adriamycin and *N*-trifluoroacetyladriamycin-14-valerate. *Cancer Treat. Rep.,* 62 (1978) 111–116.