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Short Communication

# High-performance liquid chromatographic determination of mitoxantrone in liposome preparations using solid-phase extraction and its application in stability studies

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**Abstract**

A method for the determination of the entrapped mitoxantrone in liposome preparations was developed. The method consists of a solid-phase extraction procedure followed by HPLC analysis. A $C_{18}$ cartridge was used for solid-phase extraction and 0.5 *M* methanolic HCl was used for elution. The extraction demonstrated a good separation of the mitoxantrone from the phospholipid. A $C_{18}$ column and a mobile phase containing acetonitrile–0.01 *M* monopotassium phosphate (40:60) with the pH adjusted to 3.0 with orthophosphoric acid were employed. The detection wavelength was 242 nm. The HPLC method was stability indicating and was applied to determine the degradation of the entrapped mitoxantrone in liposomes. A pseudo-first-order reaction was found for the degradation of the entrapped mitoxantrone. The half-life of the mitoxantrone decreased with increasing pH of the medium. The results demonstrate that the proposed method is satisfactory for the determination of the stability of mitoxantrone in liposome preparations.

## 1. Introduction

Liposomes are widely used as carriers for anticancer drugs [1]. Mitoxantrone, an anthracene derivative, shows significant antitumour activity in advanced breast cancer, leukaemia and lymphoma [2]. Mitoxantrone-containing liposomes have been developed [3] and are expected to increase the antitumour activity for some modestly active or inactive cancer systems. HPLC methods together with various sample clean-up procedures for the determination of mitoxantrone in solutions or biological fluids have been introduced [4–16]. However, there appears to be no HPLC method available for the determination of the entrapped mitoxantrone in liposome preparations.

This study was conducted to develop a method for the determination of the entrapped mitoxantrone in liposomes by HPLC using solid-phase extraction. The application of this method in stability tests for the entrapped mitoxantrone in liposome preparations was also studied. The use of solid-phase extraction as a sample clean-up procedure for the liposome preparations offers the advantage of avoiding emulsification of the sample during extraction. As the main component of liposome is phospholipid, which is a good emulsifying agent, the liposome preparation may easily form an emulsion when liquid–liquid extraction is used in the sample clean-up procedure.

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## 2. Experimental

### 2.1. Materials

Mitoxantrone was obtained from Kingdom Pharmaceutical (Taipei, Taiwan). Phosphatidylcholine (from fresh egg yolk, Type XI E) and cholesterol were purchased from Sigma (St. Louis, MO, USA). Dicetyl phosphate was obtained from Pharmacia (Uppsala, Sweden). Water-soluble siliconizing fluid (AquaSil) was purchased from Pierce (Rockford, IL, USA). Propyl paraben (internal standard) was obtained from Fluka (Buchs, Switzerland). All chemicals were of analytical-reagent grade and all solvents were of HPLC grade.

### 2.2. Preparation of liposomes

Multilamellar liposomes were prepared by a method described previously [3]. Phosphatidylcholine, cholesterol and dicetyl phosphate at a molar ratio of 1.6:1.0:0.15 were dissolved in chloroform in a 50-ml round-bottomed flask and dried in a rotary evaporator under reduced pressure at 37°C to form a thin film on the flask. The desired concentration of mitoxantrone in 0.9% sodium chloride solution was added to the film. Multilamellar liposomes were formed by constant vortex mixing for 5 min on a vortex mixer (Thermolyne, Dubuque, IA, USA). The mitoxantrone-containing liposomes were separated from the unentrapped mitoxantrone by dialysis (Spectra/Por 2, molecular mass cut-off 12 000–14 000; Spectrum, Los Angeles, CA, USA) for 24 h until the dialysate was free from mitoxantrone.

### 2.3. Extraction procedure

An Aspec system (Gilson, Villiers le Bel, France) equipped with 3-ml Supelclean LC-18 cartridges (Supelco, Bellefonte, PA, USA) was used for sample extraction. The cartridge was preconditioned by washing with 1 ml of methanol and 1 ml of water. One volume of mitoxantrone-containing liposomes was dissolved in three volumes of absolute ethanol and 0.5 ml of the sample was applied to the cartridge. After the sample had passed through the cartridge followed by washing with 1 ml of water, the drug was eluted with 1.0 ml of 0.5 *M* methanolic HCl. The elutate was stored at −70°C for HPLC analysis.

### 2.4. Recovery study

One volume of a mixture of a known amount of mitoxantrone standard solution and empty liposomes was added to three volumes of absolute ethanol and mixed well to dissolve the liposomes to give a phospholipid solution. The resultant concentration of the solution was 10 $\mu$g/ml for both mitoxantrone and phospholipid. A 0.5-ml volume of this solution was used for extraction immediately and chromatographed in triplicate. Recoveries were calculated by comparing the peak heights of the spiked samples with those for the standards.

In order to test for the presence of phospholipids in the eluate, the method of Schiefer and Neuhoff was used [17]. This method involved fluorimetric determination of phospholipids by means of rhodamine complexation. This method is fairly sensitive, allowing the measurement of phospholipid in the range 0.1–100 $\mu$g.

### 2.5. High-performance liquid chromatography

The HPLC apparatus consisted of two solvent-delivery pumps (Model 880-PU; Jasco, Tokyo, Japan), a solvent-mixing module (Model 880-30 Jasco), a variable-wavelength UV–Vis detector (Model 870-UV; Jasco), an on-line degasser (ERC-3511; Erma, Tokyo, Japan), an autosampler (Model 851-AS; Jasco), a system controller (Model 801-SC; Jasco) and an integrator (Chromatocorder 12; SIC, Tokyo, Japan). The column employed for analysis was reversed-phase column (25 cm × 4.6 mm I.D.) packed with LiChrospher RP-18, 5 $\mu$m (Merck, Darmstadt, Germany). The mobile phase was acetonitrile–0.01 *M* monopotassium phosphate (40:60, v/v) with the pH adjusted to 3.0 with orthophosphoric acid. The detector was operated at 242 nm with a sensitivity range of 0.005 AUFS. The attenuation of the integrator was set

at 4. The flow-rate was 1.0 ml/min and the temperature was ambient. The internal standard was propyl paraben.

### 2.6. Stability study

The buffers used for the stability measurements were pH 3.7 acetate buffer (0.1 *M* acetic acid and sodium acetate), pH 5.8 phosphate buffer (0.5 *M* monopotassium phosphate and disodium phosphate), pH 7.4 phosphate buffer (0.5 *M* monopotassium phosphate and disodium phosphate) and pH 9.6 carbonate buffer (1.0 *M* sodium hydrogencarbonate and sodium carbonate). The ionic strength of the buffers was adjusted with sodium chloride to a value equivalent to that of 0.9% sodium chloride.

The liposome preparations were dispensed and sealed into the AquaSil-treated glass vials. The samples were stored at 37°C and removed at designated times for the measurement of mitoxantrone concentration. To ensure that the method was stability indicating, the intact mitoxantrone in solution was heated at 80°C for 36, 6 and 0.5 h at pH 3.6, 7.4 and 9.6, respectively, to accelerate degradation of the drug [18,19].

### 2.7. Calibration graph

Accurately weighed mitoxantrone was dissolved in water to make a stock standard solution. Suitable dilutions of the stock standard solution to the concentration range 12.5–200 ng/ml and triplicate injections into the HPLC system were made. The calibration graph was constructed by plotting the mean peak-height ratios of mitoxantrone to propyl paraben against mitoxantrone concentration. Each run with the samples, which involved about 24 injections, was carried out with a new calibration graph constructed using the stock standard solution.

## 3. Results and discussion

Fig. 1 shows the chromatograms of mitoxantrone after heating at 80°C for 36, 6 and 0.5 h at pH 3.6, 7.4 and 9.6, respectively. The retention time was 4.2 min for mitoxantrone, 8.4 min for the degradation product of mitoxantrone and 11.4 min for propyl paraben. No degradation of propyl paraben was observed in this study. It is clear that the eluted peaks of the mitoxantrone, its degradation product and propyl paraben were well separated, demonstrating that the HPLC method is stability indicating.

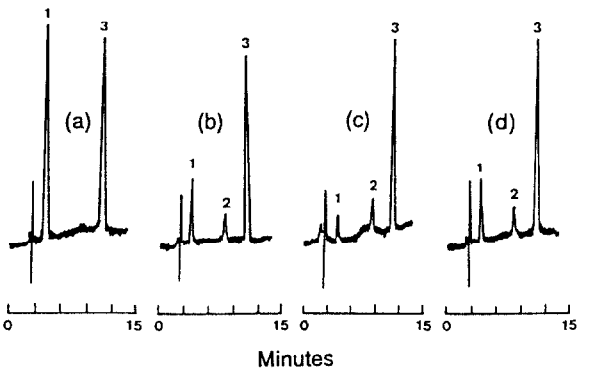


Fig. 1. HPLC of (1) mitoxantrone, (2) mitoxantrone degradation product and (3) propyl paraben. (a) Standard mitoxantrone (100 ng/ml) and propyl paraben (150 ng/ml); mitoxantrone (b) at pH 3.6 heated at 80°C for 36; (c) at pH 7.4 heated at 80°C for 6 h; (d) at pH 9.6 heated at 80°C for 30 min.

Nine standard solutions of mitoxantrone in the concentration range 12.5–200 ng/ml were analysed. The calibration graphs were analysed by linear least-squares regression and showed a correlation coefficient of 0.999. The relative standard deviations in within-day and between-day ($n = 10$) assays were 0.51 and 1.22%, respectively, at a concentration of 100 ng/ml.

The recovery of mitoxantrone from phospholipid solution using the solid-phase extraction method for five preparations showed an average of 83.5–88.0%. No trace of phospholipid was found in the eluate after solid-phase extraction. This indicated that the mitoxantrone was well separated from the phospholipid.

For the stability study, the logarithm of the concentration of residual mitoxantrone was plotted against time for the entrapped mitoxantrone in liposomes at pH 3.6, 5.8, 7.4 and 9.6 when stored at 37°C as shown in Fig. 2. It is clear that the degradation of mitoxantrone followed a pseudo-first-order reaction. The degradation rate of the mitoxantrone increased with increasing

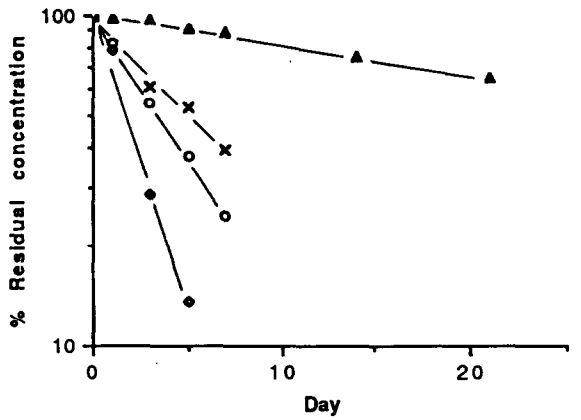


Fig. 2. Concentration–time plots of entrapped mitoxantrone in liposomes at pH (△) 3.6, (×) 5.8, (○) 7.4 and (◇) 9.6 stored at 37°C.

pH of the medium. The possible reaction for the degradation of mitoxantrone may be due to the oxidation of the phenylenediamine moiety of mitoxantrone to form a quinone diimine and then cyclization to yield the degradation product [20,21]. This reaction is significant at higher pH. Fig. 3 shows the half-life of the entrapped mitoxantrone at pH 3.6, 5.8, 7.4 and 9.6 when stored at 37°C. The most stable liposome preparation showed a half-life of 75 days at pH 3.6. In contrast, it was only 3.9 days for the less stable preparation at pH 9.6.

In conclusion, the method described here is accurate and precise for the determination of the entrapped mitoxantrone in liposome preparations, and the solid-phase extraction procedure is capable of separating mitoxantrone from liposomes. This method was successfully applied to a stability study of the entrapped mitoxantrone in liposomes.

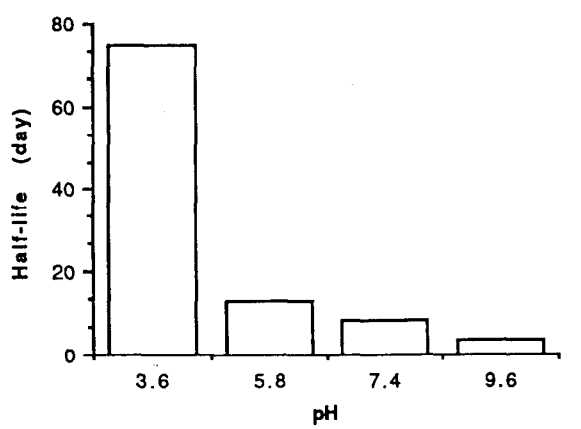


Fig. 3. Half-lives of entrapped mitoxantrone in liposomes at pH 3.6, 5.8, 7.4 and 9.6 stored at 37°C.

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