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Note

Determination of adriamycin in liposomes by high-performance liquid chromatography using a fluorescence detector

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Adriamycin in biological samples has been determined by bioassay[1], fluorescence methods[2–6], isotope methods[1,7,8] and high-performance liquid chromatography (HPLC)[9–12]. Of these methods, HPLC is the most useful because it suffers least from effects of contaminating materials.

We have examined HPLC using a fluorescence detector for the determination of adriamycin and have applied the method to the measurement of adriamycin in liposomes, which is important in cancer chemotherapy.

EXPERIMENTAL

Reagents

Adriamycin hydrochloride and adriamycinone were kindly donated by Kyowa Hakko Kogyo Co. (Tokyo, Japan) and daunomycin was by Meiji Seika Co. (Tokyo, Japan). Egg lecithin was purified from chicken egg yolk by the method previously reported[13]. Cholesterol was purchased from Nakarai Kagaku Co. (Tokyo, Japan) and recrystallized. Dicethyl phosphate was purchased from Sigma (St. Louis, Mo., U.S.A.) and stearylamine from Tokyo Kasei Kogyo Co. (Tokyo, Japan).

HPLC

A Hitachi Model 635A high-performance liquid chromatograph was connected to a Hitachi Model 650-10S high-sensitivity fluorescence detector, and the results were recorded on a Hitachi Model 056 recorder. The stationary phase was Zorbax Sil (5 μm) packed in a stainless-steel tube (150 × 4.6 mm I.D.). The mobile phase was 3.8% sodium acetate (pH 4.5) in isopropanol[10] at a flow-rate of 1.0 ml/min. Measurements were made at an excitation wavelength of 470 nm and an emission wavelength of 585 nm with an internal standard (daunomycin).

Determination of adriamycin in liposomes

Egg lecithin + cholesterol (neutral, molar ratio 7:2) or the same mixture supplemented with dicetyl phosphate (negatively charged, molar ratio 7:2:1) or stearylamine (positively charged, molar ratio 7:2:1) were dissolved in 5 ml of chloroform in a round-bottomed flask. Rotary evaporation at 30° under nitrogen *in vacuo* resulted in the formation of a thin film, which was immediately dispersed under nitrogen in 2 ml of adriamycin hydrochloride solution (1 mg/ml in sterilized saline). The suspension was sonicated under nitrogen for 30 min at 0° using Bransonic sonicator (bath type, 98 V) and then washed twice with saline at 100,000 *g* for 30 min. The sonicated liposomes entrapping adriamycin were dried *in vacuo* and were destroyed by the addition of chloroform, and then the chloroform was evaporated *in vacuo*. By centrifugation at 3000 *g* for 20 min, the supernatant was diluted with the chromatographic elution mixture containing an internal standard (daunomycin), and 5-μl samples were injected for HPLC.

RESULTS AND DISCUSSION

The determination of adriamycin by HPLC with a fluorescence detector, with excitation at 470 nm and emission at 585 nm, was found to suffer least from effects of the contaminating blank, and the separation of adriamycin, adriamycinone and daunomycin was good (Fig. 1).

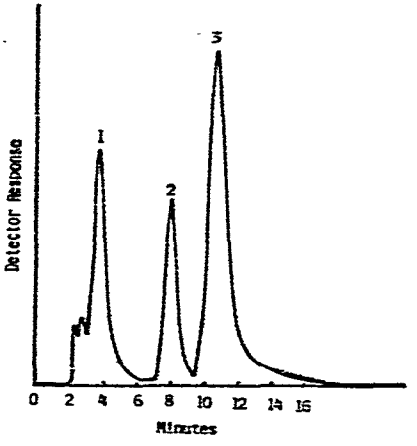

Fig. 1. High-performance liquid chromatogram: 1 = adriamycinone; 2 = daunomycin; 3 = adriamycin. Apparatus: Hitachi Model 635A high-performance liquid chromatograph. Column, Zorbax Sil (150 × 4.6 mm I.D.) at room temperature; mobile phase, 3.8% sodium acetate in isopropanol; flow-rate, 1 ml/min. Detector: Hitachi Model 650-10S fluorescence spectrophotometer (excitation, 470 nm; emission, 585 nm); sensitivity, 10; fine, 6; pen range, 5 mV.

When a standard sample of adriamycin was used, the calibration graph was linear over the range 0–1.2 μg/ml when the ratio of the peak area to that of daunomycin was used, and was linear over the range 0–0.8 μg/ml when the ratio of peak heights was used (Fig. 2).

Entrappment of adriamycin was 1.2% in positively charged liposomes, 2.7% in neutral liposomes and 35.5% in negatively charged liposomes (Table I).

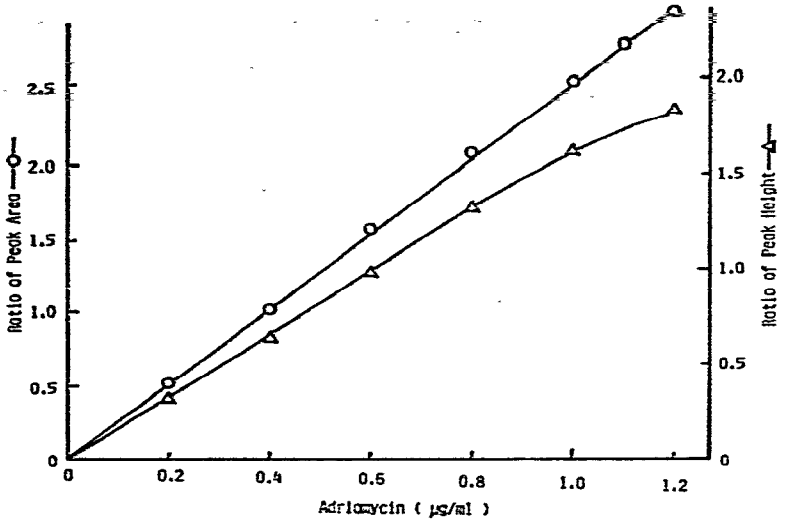

Fig. 2. Calibration graphs for adriamycin: ○, ratio of peak area to that of daunomycin; △, ratio of peak height to that of daunomycin.

TABLE I

EFFICIENCY OF THE ENTRAPPMENT OF ADRIAMYCIN IN LIPOSOMES AS DETERMINED BY HPLC

A 2-ml volume of adriamycin hydrochloride solution (1 mg/ml) was entrapped in liposomes composed of 15 mg of egg lecithin (EL) supplemented with 2.2 mg of cholesterol (CHOL) and 0.8 mg of stearylamine (STEAR) or 1.5 mg of dicetyl phosphate (DICE). The molar ratio of each set of phospholipid, cholesterol and charged lipid was 7:2:1.

Composition of liposomes	*Proportion incorporated (%) (mean ± S.D., n = 4)*	*Incorporation (μg/mg EL)*
Neutral (EL, CHOL)	2.7 ± 0.8	3.6
Positively charged (EL, CHOL, STEAR)	1.2 ± 0.6	1.6
Negatively charged (EL, CHOL, DICE)	35.5 ± 15.2	37.8

With UV detection at 235 nm, several peaks were obtained but they were disturbed by many unknown substances, and a spectrophotometric determination at 490 nm gave optimal selectivity but was not of high sensitivity. However, using the fluorescence detector with excitation at 470 nm and emission at 585 nm, no interferences were found and a high sensitivity was obtained. Using our method, the separation of adriamycin, adriamycinone and daunomycin was good and reproducible. Therefore, it is expected that this method will be applicable to the determination of adriamycin in biological samples containing anthracycline metabolites.

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