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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR PHARMACOKINETIC STUDIES ON THE NEW ANTHRACYCLINE 4-DEMETHOXYDAUNORUBICIN AND ITS 13-DIHYDRO DERIVATIVE

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SUMMARY

The anthracyclines are a group of antitumoral antibiotics with significant clinical efficacy. Among the new anthracycline derivatives, 4-demethoxydaunorubicin showed interesting biological properties in terms of both spectrum of activity and therapeutic index and was recently introduced in clinical trials. The present paper describes the analytical method developed to investigate the pharmacokinetics of this derivative.

The method consists of the extraction of 4-demethoxydaunorubicin and its 13-dihydro metabolite from plasma with chloroform—1-heptanol (9:1) and re-extraction with 0.3 *M* phosphoric acid, separation by high-performance liquid chromatography and quantification by sensitive fluorescence detection.

Plasma level curves obtained from cancer patients treated with the drug are shown.

INTRODUCTION

The anthracycline antibiotics are a group of chemotherapeutic agents with significant antitumor activity.

Daunorubicin and doxorubicin are the best known drugs of this family. In the Research and Development Laboratories of Farmitalia Carlo Erba, new anthracycline analogues have been synthesized and tested in an attempt to obtain new anticancer agents with improved efficacy and therapeutic index. 4-Demethoxydaunorubicin (I) (Fig. 1) showed interesting biological properties [1—3] and is currently under extensive clinical investigation. In order to investigate the pharmacokinetic properties of this new compound in man, a specific, sensitive and accurate analytical method is required to measure con-

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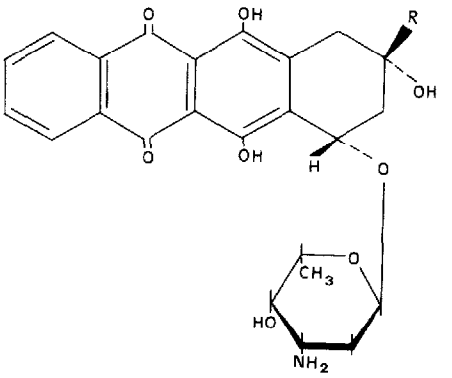

4-demethoxydaunorubicin (I) $R=COCH_3$

13-dihydro-4-demethoxydaunorubicin (II) $R=CH(OH)CH_3$

Fig. 1. Structure of 4-demethoxydaunorubicin (I) and of 13-dihydro-4-demethoxydaunorubicin (II).

centrations of the unchanged drug and of its main metabolite, the 13-dihydro derivative, in biological fluids.

This paper describes in detail this method and presents some results of its application on biological samples obtained from cancer patients treated with the drug at different dosage schedules.

The method is based on previous procedures reported by other workers [4, 5] in this field, using high-performance liquid chromatography (HPLC) coupled with a highly sensitive detection system such as ultraviolet (UV) or fluorimetric detection. The combination of highly efficient separation and highly sensitive detection proved capable of overcoming the difficulties of determining even very low concentrations of drug at long intervals after administration.

EXPERIMENTAL

Chemicals

Compound I, its 13-dihydro derivative (II) and doxorubicin (internal standard) were supplied by the Chemical Research and Development Laboratories of Farmitalia Carlo Erba. Desipramine chloride was from Prodotti Gianni (Milan, Italy); all other chemicals and solvents were from Farmitalia Carlo Erba (Analytical Division), used without further purification.

Instrumentation

A Spectra-Physics (Santa Clara, CA, U.S.A.) high-performance liquid chromatograph, Model SP 3500, equipped with a Rheodyne injection system, Model 7120, and a 170-μl sample loop (laboratory made) was used. The chromatographic detectors were a Laboratory Data Control (Riviera Beach, FL, U.S.A.) UV Monitor III, Model 1203 (cell volume 10 μl, path length 10 mm) at a fixed wavelength, and a Schoeffel (Westwood, NJ, U.S.A.) fluorimetric detector, Model FS 970 (5 μl cell volume). They were interfaced to a Spectra-

Physics laboratory data system, Model SP 4000, and the data were recorded on a terminal printer plotter SP 4050 (Spectra-Physics).

The column used was a μBondapak Phenyl (30 cm × 2 mm I.D., particle size 10 μm) (Waters Assoc., MA, U.S.A.). The column and the mobile phase were thermostatically controlled at 23 ± 0.5°C. A pellicular ODS precolumn (Whatman, Clifton, NJ, U.S.A.) was used.

All glassware was silanized before use by treatment with dichlorodimethylsilane–toluene (7:93) followed by washing with methanol and chloroform.

Drug administration

The drug was administered in distilled water intravenously, over a period of 1–5 min, or orally in capsules at doses of 15 mg/m^2 and 45 mg/m^2 body surface area, respectively.

Plasma samples from cancer patients

Samples of 5–7 ml (heparinized) of venous blood were collected before and at appropriate times after drug administration. These were immediately centrifuged, and the plasma was separated and frozen at –20°C until analysed.

Spiking of plasma samples

To determine the precision and accuracy of the method, blank plasma samples were spiked with appropriate amounts of the drug, its 13-dihydro derivative and the internal standard. These substances were dissolved as hydrochloride salts in distilled water containing 10 μg/ml desipramine. The solutions were prepared weekly and stored at 4°C. The stability of the substances was tested daily by HPLC analysis.

The samples were submitted to the analytical procedure described below.

Analytical procedure

Extraction. To 1-ml plasma samples, 10–40 ng of doxorubicin (internal standard) in 0.1 ml of water were added. The samples were carefully mixed with 2 ml of borate buffer, pH 8.4 (ionic strength, μ = 0.05) in a 15-ml glass-stoppered test tube and extracted with 10 ml of a chloroform–1-heptanol (9:1) mixture by mechanical shaking for 40 min. After centrifugation at 1200 *g* for 10 min the upper aqueous layer was removed by aspiration. The lower organic phase was transferred to another test tube and re-extracted with 0.3 ml of 0.3 *M* phosphoric acid, containing 10 μg/ml desipramine, in order to avoid adsorption losses, for 10 min. The aqueous phase was again transferred to a centrifuge test tube containing 2 ml of hexane and centrifuged. A 170-μl portion of the aqueous phase was injected into the chromatographic column.

Chromatographic procedure and quantitation. The mobile phase consisted of an isocratic mixture of acetonitrile–0.05 *M* KH_2PO_4 (35:65). The flow-rate was 0.4 ml/min. The fluorimeter was set at 254 nm excitation wavelength and 550 nm (filter) emission wavelength. Compounds I and II can also be determined using an UV detector with high sensitivity at a fixed wavelength of 254 nm.

Quantitation was obtained by peak area measurement with the computer integrator.

RESULTS AND DISCUSSION

Extraction procedures

The extraction of anthracyclines using chloroform and 1-pentanol or heptanol has been extensively studied [6, 7]. The degree of extraction depends greatly on the pH of the aqueous phase. For compounds I and II we found the optimum extraction was at pH 8.4, close to the optimum value (pH 8.6) for doxorubicin, used as internal standard. Interference from aglycone metabolites is avoided since these compounds are not re-extracted into the acidic phase.

Chromatographic separation

Separation of anthraquinone glycosides by liquid chromatography has already been described in detail [8]. On the basis of these studies and our own we found that the chromatographic system used gave good results regarding selectivity and efficiency.

We analyzed about 250 samples without any loss in the efficiency of the column. This long column life is due to the use of a precolumn which was refilled with new pellicular ODS every week.

To obtain the maximum detector response, we found that the best mobile phase was a mixture with a relatively high concentration of acetonitrile and a correspondingly low flow-rate.

Fig. 2A shows the chromatogram of a plasma extract from a cancer patient treated with compound I, whereas Fig. 2B shows the chromatogram of a blank plasma extract. No peaks corresponding to the retention times of the two compounds are present in this latter chromatogram.

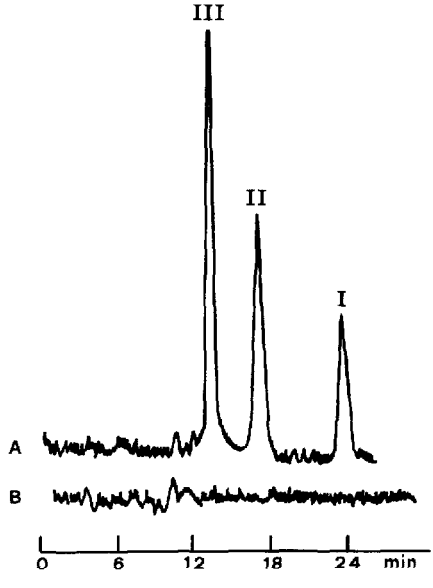

Fig. 2. Chromatograms of (A) plasma extract from a cancer patient 2 h after an intravenous dose (15 mg/m^2) of 4-demethoxydaunorubicin, and (B) blank plasma extract. Peaks: I = 4-demethoxydaunorubicin (8 ng/ml), II = 13-dihydro-4-demethoxydaunorubicin (12 ng/ml), III = doxorubicin (internal standard) (40 ng/ml). Chromatographic conditions are detailed in the text.

Selectivity and sensitivity

Compounds I and II can be determined by using both UV and fluorescence detectors because of their high absorbance at 254 nm. Thus, with a UV detector at a fixed wavelength of 254 nm we obtained the same results as with a fluorimetric detector in terms of sensitivity. We tried the UV detector for the determination of plasma levels, but the chromatograms of some samples showed peaks that interfered with the drug and its metabolite. These interferences were probably due to other drugs administered to patients together with compound I. To avoid these interferences we preferred the fluorimetric detector since it proved more selective.

The excitation wavelength used, 254 nm, gave better results in terms of response/noise ratio than the wavelength usually used, 470 nm.

Quantitative determination

The evaluation of compounds I and II is based on the use of the internal standard (doxorubicin) added in known amounts to the plasma samples. The relative weight ratio (RWR) values were obtained by assaying blank plasma samples spiked with known amounts of the two compounds, by the equation

$$\mathrm{RWR} = \frac{A_s}{A_{is}} \cdot \frac{C_{is}}{C_s}$$

where

A_s = peak area of I or II
A_{is} = peak area of internal standard
C_{is} = amount of internal standard in the sample (ng/ml)
C_s = amount of I or II in the sample (ng/ml).

The RWR values are constant for each compound in a given chromatographic condition and are used for the determination of unknown plasma samples according to the equation

$$C_s = \frac{A_s}{A_{is}} \cdot \frac{C_{is}}{\mathrm{RWR}}$$

The RWR values were 2.43 and 2.61, respectively, for compounds I and II. The coefficients of variation of the RWR values provide an estimate of the precision of the analytical method and were 5.95 and 6.3% (n = 20). The linearity gave regression coefficients of r = 0.995 and r = 0.997 for compounds I and II,

TABLE I

ACCURACY OF 4-DEMETHOXYDAUNORUBICIN DETERMINATIONS

Amount added (ng/ml)	No. of samples	Mean amount found (ng/ml)	Coefficient of variation (%)
40.0	4	40.76	3.6
20.0	4	19.67	4.0
10.0	4	9.64	5.69
5.0	4	4.98	6.8
		Average:	5.1

TABLE II

ACCURACY OF 13-DIHYDRO-4-DEMETHOXYDAUNORUBICIN DETERMINATIONS

Amount added (ng/ml)	No. of samples	Mean amount found (ng/ml)	Coefficient of variation (%)
40.0	4	40.21	3.3
20.0	4	20.13	2.41
10.0	4	9.94	3.44
5.0	4	5.01	8.58
		Average:	4.53

respectively, in the concentration range 5—30 ng/ml. The data for the accuracy test are given in Tables I and II; the coefficients of variation were 5.1% and 4.5%, for compounds I and II, respectively.

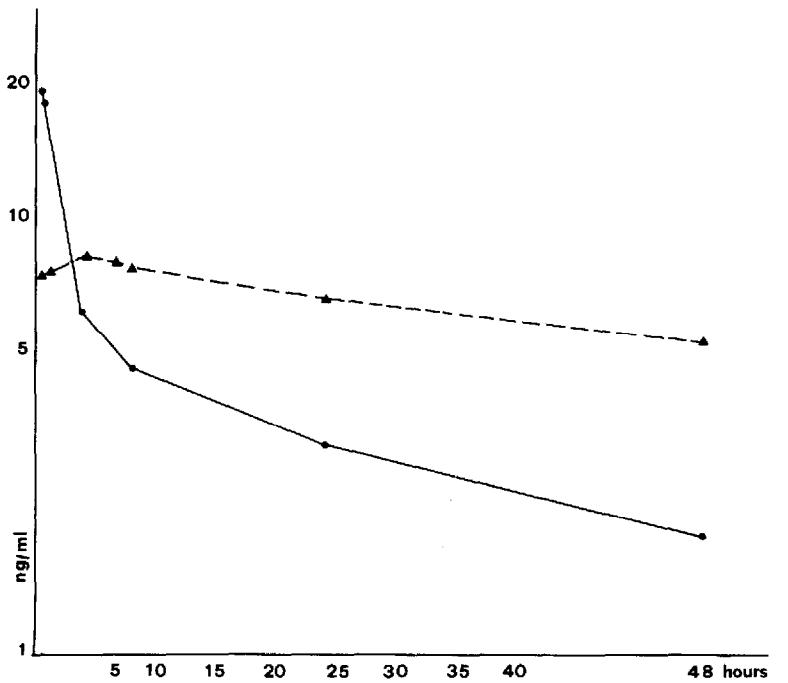

Fig. 3. Plasma levels of compounds I (•) and II (▲) in a cancer patient after intravenous administration of 15 mg/m² 4-demethoxydaunorubicin (I).

Plasma samples from patients

The method described here was applied to several plasma samples from cancer patients who received compound I. The plasma levels of the unchanged drug and of its 13-dihydro metabolite after intravenous (15 mg/m²) and oral (45 mg/m²) administration are given in Figs. 3 and 4, respectively. With this method the aim of measuring plasma levels of compounds I and II with a good degree of accuracy and high sensitivity has been achieved. Generally the sensiti-

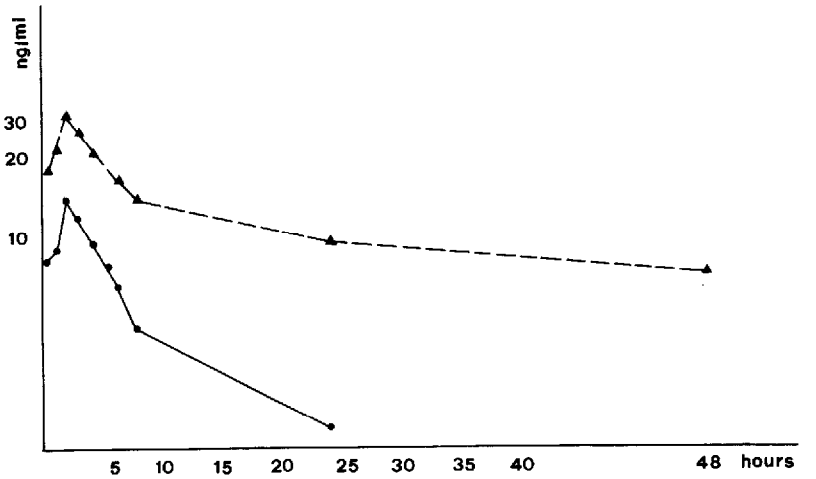

Fig. 4. Plasma levels of compounds I (•) and II (▲) in a cancer patient after oral administration of 45 mg/m² 4-demethoxydaunorubicin (I).

vity of the analytical method for anthracyclines is particularly important because of the unusual plasma level profiles of these drugs, which are characterized first by a rapid decrease to values below 10 ng/ml, followed by a very slow terminal phase with plasma half-live of about 1—2 days. This problem is particularly important with very potent anthracyclines such as compound I administered at doses substantially lower than doxorubicin, daunorubicin and 4′-epidoxorubicin.

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