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Note

Determination of 4′-epidoxorubicin and its 13-dihydro derivative in human plasma by high-performance liquid chromatography with fluorescence detection

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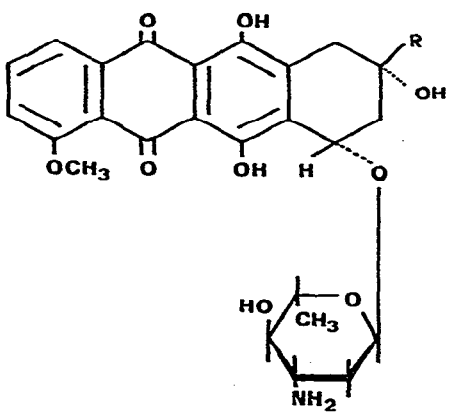
4′-Epidoxorubicin (I) is a new anthraquinone glycoside with antit mor activity synthesized in the Farmitalia Carlo Erba Laboratories [1—6]. For pharmacokinetic studies an analytical method was required for the determination of plasma levels of I and one of its metabolites, 13-dihydro-4′-epidoxorubicin (II) (Fig. 1). This paper describes a method, based on previously reported procedures [7,8] for the extraction of anthraquinone glycoside, which allows the



(I) 4′-Epidoxorubicin R= $COCH_2OH$

(II) 13-Dihydro -4′-epidoxorubicin R= $CH(OH)CH_2OH$

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

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determination of plasma levels of I and II with a detection limit of 3—4 ng/ml. Data on plasma levels of I and II in a cancer patient, following a 70 mg/m^2 intravenous dose of I, are reported.

EXPERIMENTAL

Chemicals and solvents

Compounds I and II were supplied by the Chemical Research and Development Laboratories of Farmitalia Carlo Erba (Milan, Italy); desipramine hydrochloride was from Prodotti Gianni (Milan, Italy), 1-heptanol (analytical grade) from Merck-Schuchardt (Munich, G.F.R.) and acetonitrile (Lichrosolv) was from Merck (Darmstadt, G.F.R.). All other chemicals and solvents (spectrophotometric grade) were purchased from Farmitalia Carlo Erba.

Stock solutions of I and II contained 20 μg of each substance and 10 μg of desipramine·HCl per ml water. These solutions were stored at 4°C. From them, working solutions at a concentration ranging between 20 and 1000 ng/ml for I and 15 and 250 ng/ml for II, were prepared weekly by suitable dilution with water and stored at 4°C when not in use.

Instrumentation

A Spectra-Physics (Santa Clara, CA, U.S.A.) high-pressure liquid chromatograph, Model SP 3500 B, equipped with a Rheodyne injection system, Model 7120, and a sample loop of 170 μl (laboratory made) was used. The column (prepacked, 25 cm × 4 mm I.D.) contained Partisil ODS, reversed-phase 10 μm microparticulate (Whatman Inc., Clifton, NJ, U.S.A.) and was preceded by a C_{18} precolumn (Co:Pell ODS, 7 cm × 2.1 mm I.D.; Whatman), and maintained at 25 ± 1°C by a water jacket.

The chromatograph was coupled with a Schoeffel (Westwood, NJ, U.S.A.) fluorescence detector, Model FS 970; excitation and emission were set at 470 and 580 nm, respectively. For the peak area calculations, the detector was interfaced to a laboratory data system SP 4000 (Spectra-Physics) and the data recorded on a terminal printer plotter SP 4050 (Spectra-Physics).

The mobile phase was an isocratic mixture of acetonitrile and 0.03 *M* phosphoric acid (40:60, v/v) with a constant flow-rate of 0.4 ml/min. With the detector sensitivity set at 0.2 μA, full-scale response was obtained by 45 ng of compound I.

Extraction procedure

Known amounts of I and II were added to aliquots of 2 ml of human plasma diluted with 1 ml of pH 8.4 phosphate buffer in a 15-ml glass stoppered test tube. The mixtures were extracted with 10 ml of chloroform—1-heptanol (9:1) by mechanical shaking for 30 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 absorption losses, for 10 min. The aqueous phase was transferred again into a centrifuge test tube containing 2 ml of hexane and centrifuged. A portion of 0.17 ml of the aqueous phase was injected into the chromatographic column.

Precision and accuracy

The peak areas obtained by analysing blank plasma samples spiked with known amounts of I and II were divided by these values to obtain the specific areas. The mean specific areas were used to calculate the amount of I and II in unknown samples. The coefficient of variation provides an estimate of the precision of the method over the range of concentrations tested.

The accuracy of the method was investigated on replicate plasma samples containing amounts ranging between 6.46 and 167.7 ng/ml for I and 5 and 125 ng/ml for II, and estimated by the coefficient of variation.

RESULTS AND DISCUSSION

Under the chromatographic conditions reported in the experimental section, HPLC analysis showed retention times of 21.7 and 17.7 min for I and II, respectively. A chromatogram obtained from 1 ml of plasma containing 18 ng/ml I and 12 ng/ml II is presented in Fig. 2A, whereas Fig. 2B represents a chromatogram of an extracted blank plasma. No peaks corresponding to the retention times of the two compounds were found in this chromatogram. Cali-

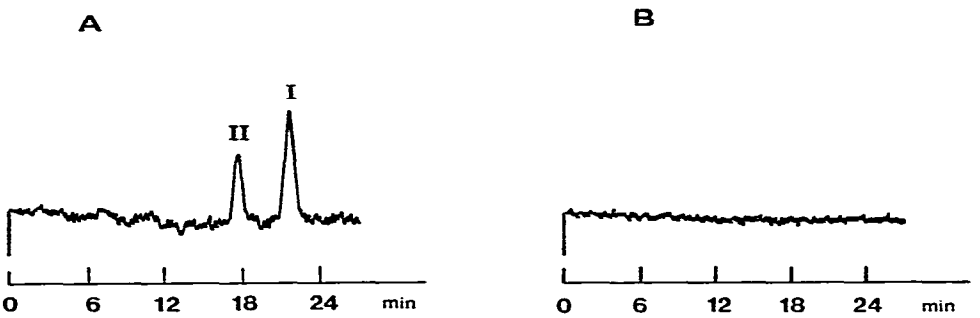

Fig. 2. Chromatograms of (A) plasma extract containing 18 ng/ml 4'-epidoxorubicin (I) and 12 ng/ml 13-dihydro-4'-epidoxorubicin (II), and (B) blank plasma extract. Chromatographic conditions are detailed in the text.

bration curves were obtained by analyzing 1-ml plasma samples spiked with 4.2, 12.9, 25.8, 64.6, 129.2, 258.5 ng of compound I (four samples for each concentration) and 2.78, 5.56, 11.12, 22.25, 44.5 ng of compound II (four samples for each concentration). The linearity gave regression coefficients of $r = 0.998$ (average coefficient of variation = 6.76%) and $r = 0.9992$ (average coefficient of variation = 4.73%) for compound I and compound II, respectively.

The good reproducibility of the method, controlled by assaying plasma samples repeated on separate occasions, appears in Tables I and II, showing average coefficients of variation of 7.15 and 7.03% for compounds I and II, respectively.

The method shows a sensitivity of 3—4 ng/ml for each compound when present in human plasma, taking as the limit of detection a value five times higher than the baseline noise. This sensitivity appears to be sufficient for therapeutic monitoring and pharmacokinetic studies. In fact, the reliability of the method has been evaluated determining the plasma levels of a cancer

TABLE I

ACCURACY OF 4'-EPIDOXORUBICIN DETERMINATIONS

Amount added (ng/ml)	No. of samples	Mean amount found (ng/ml)	Coefficient of variation (%)
167.7	4	172.18	3.16
83.85	4	81.58	5.26
41.92	4	43.08	8.43
25.85	6	25.36	8.80
12.92	6	12.83	8.92
6.46	6	6.43	8.36
		Average	7.15

TABLE II

ACCURACY OF 13-DIHYDRO-4'-EPIDOXORUBICIN DETERMINATIONS

Amount added (ng/ml)	No. of samples	Mean amount found (ng/ml)	Coefficient of variation (%)
125	4	125.19	5.55
70	4	69.82	4.69
40	4	40.57	5.53
15	4	14.79	7.21
5	6	4.85	12.19
		Average	7.03

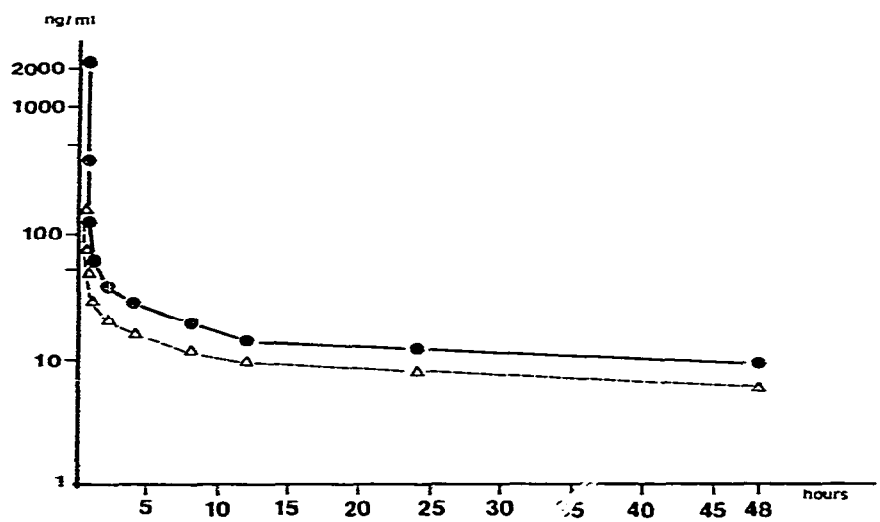

Fig. 3. Plasma levels of 4'-epidoxorubicin (●——●) and 13-dihydro-4'-epidoxorubicin (△— — —△) in a cancer patient following intravenous administration of 70 mg/m² 4'-epidoxorubicin.

patient after intravenous treatment of I at the dose of 70 mg/m². The plasma concentrations of I and II, reported from the curves in Fig. 3, exponentially decline from values as high as 100—2000 ng/ml to values around 7—10 ng/ml observed at 48 h after administration.

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