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QUANTITATION OF DAUNORUBICIN AND ITS METABOLITES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH ELECTROCHEMICAL DETECTION

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SUMMARY

A selective and sensitive high-performance liquid chromatographic method was developed for the separation and quantitation of daunorubicin and its metabolites in serum, plasma, and other biological fluids. Daunorubicin and metabolites in human plasma were injected directly into the high-performance liquid chromatography system via a loop-column to pre-extract the drugs from the plasma, and quantitated against a multilevel calibration curve with adriamycin as the internal standard. The column effluent was monitored with an electrochemical detector at an applied oxidative potential of 0.65 V and by fluorescence. Daunorubicin and four metabolites were separated and characterized by this method. In a blinded evaluation of accuracy and precision, the mean coefficients of variation were 3.8, 3.6 and 9.8% at concentrations of 150, 75 and 15 ng/ml, respectively, and blank samples gave negligible readings. The amperometric sensitivity was greater than achieved by fluorescence detection, and offers an alternative method for quantitation of these compounds. The new method has a limit of detection of less than 2 ng on column, allowing quantitation of < 10 ng/ml in plasma samples without organic extraction prior to chromatographic analysis.

INTRODUCTION

Daunorubicin is an anthracycline antibiotic which has been under clinical investigation since 1965, and has proven to be an effective antitumor agent [1, 2]. It is generally accepted that daunorubicin exerts its effects by intercalaction between the base pairs in the DNA helix in the cell nucleus, thereby preventing their replication [3, 4], although there is still uncertainty concerning the precise mode of action of the drug, its distribution and metabolism in plasma, tissue, and leukemia cells, and the possible cytotoxic role of

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intermediate metabolites. Since the majority of human pharmacokinetic studies have used assays which measure the fluorescence of the parent drug and metabolites, detection of potentially non-fluorescent metabolites was not possible, while the potential for non-fluorescent human metabolites of anthracyclines has previously been suggested [5, 6]. Therefore, the development of a sensitive and selective alternative analytical method should facilitate future investigations of anthracycline pharmacokinetics. Also, previous high-performance liquid chromatographic (HPLC) methods have generally required organic extraction of these drugs and metabolites from biological fluids by methods which are frequently inefficient (<60%) [7–11]. The present study describes a selective and sensitive isocratic HPLC method for the separation and electrochemical quantitation of daunorubicin and four potential metabolites. The new method is simple, rapid, requires no organic extraction of plasma or serum and has more sensitivity than HPLC with fluorescence methods.

MATERIALS AND METHODS

Chemicals and reagents

Daunorubicin (DNR), daunorubicinol (DOL) and daunorubicin-aglycone (DNR-AGLY) were obtained from Dr. A. Goodman, Ives Laboratories (New York, NY, U.S.A.). 7-Deoxydaunorubicinol-aglycone (7-d-DOL-AGLY) and 7-deoxydaunorubicin-aglycone (7-d-DNR-AGLY) were obtained from Drs. Nahed Ahmed and N.R. Bachur. Adriamycin (doxorubicin hydrochloride, ADR) was obtained from Adria Labs. (Columbus, OH, U.S.A.).

Acetonitrile was chromatographic grade (Burdick & Jackson Labs., Muskegon, MI, U.S.A.) and sodium acetate was analytical grade from Fisher Scientific (Pittsburgh, PA, U.S.A.).

High-performance liquid chromatography

Plasma samples (usually 250 μl) spiked with 25 ng of adriamycin (internal standard) were injected directly into a loop-column (3.9 × 2.3 mm) connected to the injector (Rheodyne 7125) of the LDC Model III high-performance liquid chromatograph, as previously described by Riley and Evans [12] for the analysis of doxorubicin. The analytical column was attached at port 2 rather than port 3 and the pump was connected at port 3 instead of port 2, providing a one-way flow of mobile phase through the injection loop-column. After the plasma sample was loaded onto the loop-column, potentially interfering substances (polar compounds, proteins, etc.) were removed by washing the loop-column (still in "load" position) with 1.0 ml of deionized water. The compounds of interest were then eluted from the loop-column and subsequently the analytical column by the mobile phase, after the injector was put in the "inject" position. After 1 min the injector was returned to the load position and the loop-column prepared to receive another sample by washing with 1–2 ml of filtered deionized water. The analytical column was a 30 cm × 3.9 mm μBondapak C_{18} 10 μm column (Waters Assoc., Milford, MA, U.S.A.), which was eluted isocratically at room temperature using a mobile phase containing acetonitrile–water–acetic acid (28:71:1) adjusted to pH 4.0 with 20% sodium acetate. Flow-rates were adjusted to 2.0 ml/min and eluates were

detected with a Gilson fluorescence detector (Spectra/Glo, Model FL-IA) and an LC-3 amperometric detector (Bioanalytical Systems, West Lafayette, IN, U.S.A.) in series. The fluorometer was equipped with a 45-μl flow cell, a 480-nm excitation filter and a 560-nm emission filter (Doxorubicin Filters, Gilson). The electrochemical detector was used at an applied oxidative potential of 0.65 V. Chromatograms were traced on a Fisher Series 5000 dual-pen chart recorder at a speed of 0.25 cm/min and at a setting of 1.0 and 0.01 V for the electrochemical and fluorescence detectors, respectively.

Precision and accuracy

To assess precision and accuracy of the new method, three 5-ml serum samples obtained from volunteers not receiving anthracyclines were spiked with 15, 75 and 150 ng/ml of DNR, DOL and DNR-AGLY. Each of these three samples were vortexed and then aliquoted into 8 equal volumes, coded, placed in random order, and frozen at −70°C, then subsequently analyzed by a single investigator not involved in the preparation of these samples. Three samples were analyzed, in duplicate, each day until all 24 samples had been analyzed. Mean values and coefficients of variation (C.V.) were computed after all samples had been analyzed. All analysis for the precision and accuracy studies were performed using electrochemical detection.

The extraction efficiency of the loop-column method was assessed by comparing peak heights of the compounds of interest injected onto the loop-column in 250 μl of plasma against the peak height of the identical amount injected directly onto the chromatographic column in methanol.

RESULTS

The new HPLC method, using a μBondapak C_{18} column eluted isocratically with a mobile phase consisting of acetonitrile—water—acetic acid (28:71:1) adjusted to pH 4.0 with sodium acetate, resolved DNR and all metabolites (Fig. 1). ADR was also resolved from all daunorubicin metabolites permitting its use as an internal standard for quantitation.

Linearity and sensitivity of the electrochemical detector were determined from serial concentrations of parent drug and metabolites. Linear calibrations were obtained for DNR, DOL and DNR-AGLY in the concentration range of 15—150 ng/ml (Fig. 2). The lowest amount of DNR detectable, defined as 2.5 times the noise level, was 2 ng which compares very favorably to other published detection methods [13—15] and was achieved at 1/10 of the maximum detector sensitivity. This amount corresponds, for the 250 μl injected into the loop column, to a concentration of ca. 8 ng/ml. As can be seen in Fig. 1, the electrochemical detector signal (10-fold lower than maximum detector settings) was considerably greater than the fluorescence signal (detector at maximum sensitivity).

Fig. 1. also demonstrates the resolving power of the system used; the following four DNR derivatives were resolved with retention times (at a flow-rate of 2.0 ml/min) of 4.8 min for DOL, 6.5 min for 2-d-DOL-AGLY, 8.0 min for DNR, 12.6 min for DNR-AGLY, and 22.3 min for 7-d-DNR-AGLY. The corresponding capacity factors (k') were 2.8, 3.8, 4.7, 7.4 and 13.1, respectively (Table I).

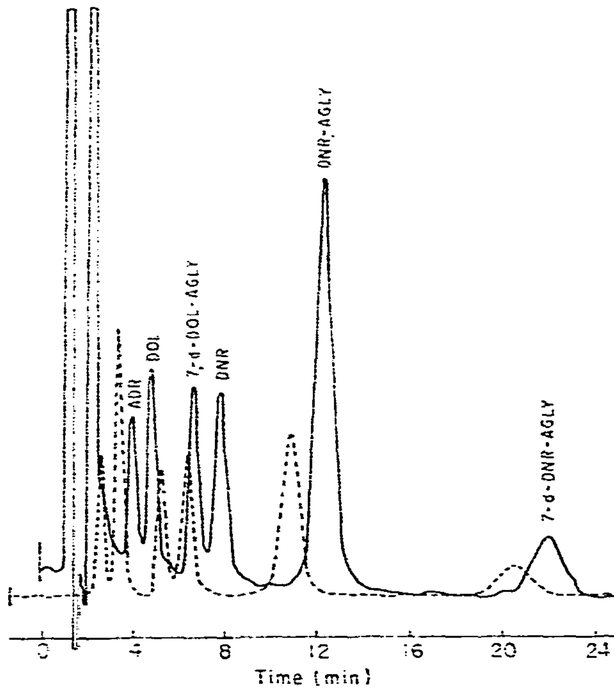

Fig. 1. Separation of a mixture of daunorubicin and metabolites in plasma by HPLC, with quantitation by fluorescence (— — —) and electrochemical (——) methods. The column was a μBondapak C_{18} (30 cm × 3.9 mm) and the solvent contained acetonitrile—water—acetic acid (28:71:1) adjusted to pH 4.0. Mobile phase flow-rate was 2.0 ml/min. The sample was a mixture of ADR (3.8 min), DOL (4.8 min), 7-d-DOL-AGLY (6.5 min), DNR (8.0 min), DNR-AGLY (12.6 min) and 7-d-DNR-AGLY (22.3 min).

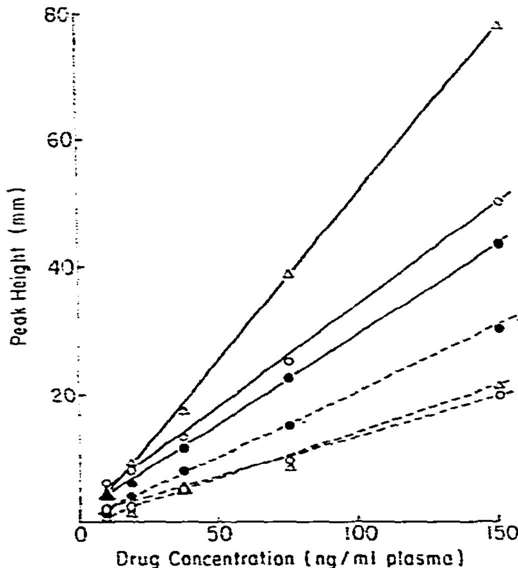

Fig. 2. Calibration graphs of peak height versus concentration for daunorubicin and metabolites in human plasma. Standard amounts of DNR (○), DOL (●), DNR-AGLY (△) were added to plasma in order to obtain drug concentrations of 4.69, 9.37, 19.75, 37.5, 75.0 and 150 ng/ml. Electrochemical (——) and fluorescence (— — —) detection.

TABLE I

DAUNORUBICIN AND METABOLITES SEPARATED BY REVERSED-PHASE HPLC

Compound	Abbreviation	Retention time (min)	k'
Adriamycin (internal standard)	ADR	3.8	2.2
Daunorubicinol	DOL	4.8	2.8
7-Deoxy-daunorubicinol-aglycone	7-d-DOL-AGLY	6.5	3.8
Daunorubicin	DNR	8.0	4.7
Daunorubicin-aglycone	DNR-AGLY	12.6	7.4
7-deoxydaunorubicin-aglycone	7-d-DNR-AGLY	22.3	13.1

TABLE II

SUMMARY OF PRECISION AND ACCURACY EVALUATION (n = 16) OF THE NEW HPLC—ELECTROCHEMICAL DETECTION METHOD FOR DAUNORUBICIN AND METABOLITES

Spiked concentration (ng/ml)	Internal standard method			Without internal standard		
	Measured (mean, ng/ml)	Accuracy (%)	Precision (C.V., %)	Measured (mean, ng/ml)	Accuracy (%)	Precision (C.V., %)
Daunorubicin						
150	151.0	100.7	3.9	151.4	100.9	3.6
75	75.1	100.1	3.6	75.6	100.8	4.6
15	16.5	110.0	12.2	16.2	108.0	11.4
Daurorubicinol						
150	152.3	101.5	4.6	148.8	99.2	2.9
75	76.0	101.3	3.4	75.1	100.1	3.4
15	16.5	110.0	7.1	16.8	112.0	18.3
DNR-aglycone						
150	149.1	99.4	3.0	148.3	98.9	1.7
75	75.8	101.1	3.7	74.9	99.9	2.4
15	16.0	106.6	10.0	16.5	110.0	13.4

A summary of the precision and accuracy studies is presented in Table II. Since an internal standard may not necessarily improve the precision or accuracy of an HPLC method [16], the necessity of the internal standard in our new method was assessed by independently computing concentrations against two separate sets of calibration curves using either peak heights (Fig. 2) or peak height ratio of compound of interest/internal standard. Both calibration curves were linear over the concentration ranges evaluated. The lowest coefficients of variation and greatest accuracy were achieved using the internal standard method (i.e., peak height ratio). The coefficients of variation were highest at the lowest concentration (7.1—12.2%), and declined to less than 5% (3.0—4.6%) at the higher concentrations. All results were within 10% of the spiked values, with the greatest deviation (106—110%) at the lowest concentrations.

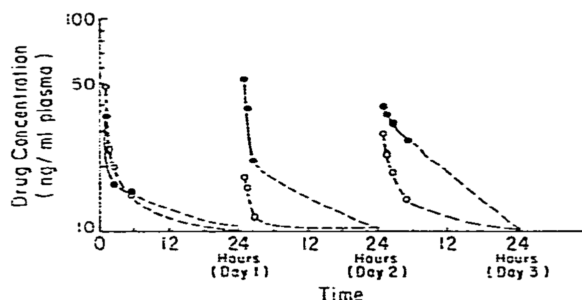

Fig. 3. Semilogarithmic plots of daunorubicin (○) and daunorubicinol (●) serum concentration versus time in a patient's plasma following 45 mg/m² daunorubicin administered intravenously on three consecutive days.

The extraction efficiency of daunorubicin and metabolites averaged 80 ± 6.2% over the concentration range evaluated.

Fig. 3 shows the plasma concentration—time profile of DNR and DOL measured by the new HPLC—electrochemical detection method in a patient with acute non-lymphocytic leukemia. Daunorubicin aglycones were not detectable in this patient's plasma.

DISCUSSION

The current study demonstrates that daunorubicin and four of its metabolites can be simultaneously separated and identified by a new HPLC technique using electrochemical detection. The procedure is simple and easy to perform. The use of the loop-column permits direct injection of plasma samples and overcomes the variability and inefficiencies of organic extraction procedures [7—11]. The electrochemical detector displayed high specificity and sensitivity, indicating the feasibility of determining anthracycline antineoplastic agents by nonfluorescent HPLC methods. The assay satisfied accepted analytical performance criteria and has been further validated by reference to a standard fluorescence detection method.

The chromatographic data from the patient's plasma samples following 45 mg/m² intravenous infusion indicate the extensive metabolism of daunorubicin following intravenous infusion, which could result in significant differences in plasma and leukemic cell exposure to parent drug and its principal cytotoxic metabolite (DOL). More extensive studies to establish the pattern of accumulation of DNR and metabolites in plasma and leukemia cells are needed, since previous work suggest that DNR metabolism may relate to clinical efficacy [17]. Previously described HPLC—fluorescence detection methods are affected by alterations of the anthracycline chromophore or changes in the quantum efficiency of chromophore fluorescence during human disposition. The new HPLC—electrochemical detection method offers a sensitive and specific alternative which is not dependent on fluorescence of parent drugs or potential metabolites.

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