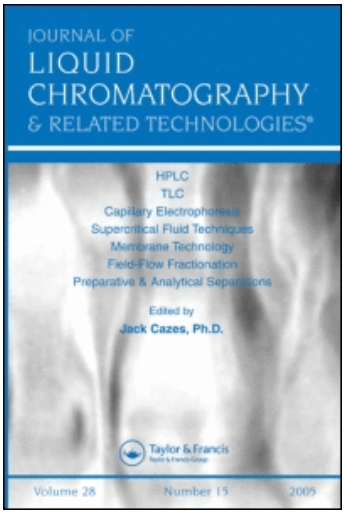

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Contemporary Detection Of 4-Demeth-Oxydaunorubicin And Its Metabolites 13-Dihydro-4-Demethoxydaunorubicin And4-Demethoxydaunorubicinone By Reverse Phase High-Performance Liquid Chromatography

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CONTEMPORARY DETECTION OF 4-DEMETHOXYDAUNORUBICIN AND ITS METABOLITES 13-DIHYDRO-4-DEMETHOXYDAUNORUBICIN AND 4-DEMETHOXYDAUNORUBICINONE BY REVERSE PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

The aim of this work was to optimize a simple analytical method for a complete pharmacological and toxicological follow up of patients treated with a new orally active daunorubicin analog, the 4-demethoxydaunorubicin. For this reason the chromatographic properties of the unchanged drug, its reduced metabolite 13-dihydro-4-demethoxydaunorubicin and its aglicone metabolite 4-demethoxydaunorubicinone have been investigated. Extraction of these compounds from biological fluids has been carried out using ethyl acetate and buthanol. Separation has been achieved in a C_{18} reverse phase column by isocratic eluition with a mobil phase consisting of acetonitrile:methanol:phosphate buffer 40:10:50, pH 4.7. Drug and metabolites can be quantitated at nanogram level by fluorescence detection.

The appearance of a further compound, identified as the 13-dihydro-4-demethoxydaunorubicinone, was noted when whole blood instead of plasma was utilized while developing the assay. Aldo-cheto reductases of red blood cells could be responsible for the reduction of the 4-demethoxydaunorubicinone in its 13-dihydroderivative.

INTRODUCTION

The antitumor activity of an antibiotic agent was first reported in 1952 (1). Twelve years later the cytotoxic activity of daunorubicin (DNR), an anthracyclin antibiotic isolated from cultures of Streptomyces Peucetius, was

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demonstrated (2). To date, anthracyclines represent the most useful group of antineoplastic antibiotics in clinical oncology: DNR and doxorubicin (DXR) are widely used in the treatment of a number of solid tumors and leukemias (3-6)

A major limitation to the use of anthracyclines (as well as of any anticancer drug) is host toxicity. In addition to the common acute dose-limiting toxicity, such as bone marrow inhibition and mucositis , anthracyclines display a cumulative (chronic) cardiac toxicity: for example, a congestive heart failure is often conseguent to DXR treatment when the total dose administered exceeds 500-600 mg/m^2 (7,8). Unfortunately, despite the efforts to understand the mechanism of anthracyclin-mediated cardiac damage, conclusions are lacking. During the last ten years remarkable efforts have been made to develop DNR and DXR analogs with reduced host toxicity and/or enhanced antitumor activity: for this reason a number of structural and stereochemical modifications have been carried out in the aminosugar and in the anthracynolone group (9-13). The 4-demethoxydaunorubicin (dmDNR, Idarubicin), whose chemical structure is shown in Figure 1, is a DNR analog currently under clinical investigation (14-17).

The knowledge of the tissue distribution and the pharmacokinetic features of a drug and its metabolites (active and/or toxic) is an important step to achieve the desired antitumor effect and to reduce the incidence and the degree of host toxicity (18). Previous publications have reported analytical methods to quantitate DNR, DXR and some analogs (including dmDNR) in biological fluids with the contemporary detection of their 13-dihydro-metabolites (19-23). Because the involvment of anthracyclines conversion into aglicone metabolites has been suggested as a possible mechanism leading to cardiac toxicity (24,25), we have developed a simple and sensitive HPLC method for the contemporary detection of dmDNR , its reduced metabolite 13-dihydro-4-demethoxydaunorubucin and its aglicone metabolites 4-demethoxydaunorubicinone and 13-dihydro-4-demethoxydaunorubicinone.

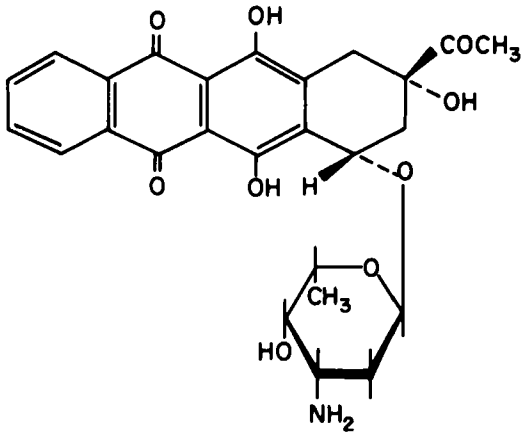

FIGURE 1. Chemical structure 4-demethoxydaunorubicin.

MATERIALS AND METHODS

Chemicals

dmDNR, its metabolites and DXR (used as internal standard) were kindly supplied by Farmitalia-Carlo Erba (Milan,Italy). Chemicals used in the extraction procedure were of reagent grade and used without further purification. Solvents used in the HPLC system were of analitical grade.

Instrumentation

A Waters HPLC system consisted of a Mod.510 solvent delivery system, a Mod.U6K sample injector and a Mod.420 AC spectrofluorimeter detector was used (Waters Assoc., Milford MA). Excitation and emission wavelengths were 254 and 530 nm respectively. The stationary phase was a μ-Bondapak C_{18} reverse-phase column, particle size 10 μm (Waters Assoc., Milford MA). The HPLC system was interfaced to a Mod.3390A reporting integrator (Hewlett Packard, Avondale PA).

Analytical Procedures

100 µg/ml stock solutions of each compound have been prepared in methanol and stored at -20°C. Fresh solutions were prepared every two weeks. In order to avoid loss of compounds all the glassware were silanized with a 1% aqueous solution of Prosil 28 (PCR Inc., Gainesville FA). Solutions containing the anthracyclines were protected from light.

Extraction of dmDNR and its metabolites from biological fluids has been performed as follow: 1.3 µg of DXR (internal standard) and 1.0 ml borate buffer of pH 8.5 were added to 1.0 ml sample (whole blood, plasma or urine) which was previously spiked with different concentrations of dmDNR, 13-dihydro-4-demethoxydaunorubicin (M I) and 4-demethoxydaunorubicinone (M II). Extraction was carried out two times with 5.0 ml of a mixture ethyl acetate:buthanol (3:2) at room temperature and the aqueous phase was re-extracted with 1.5 ml of ethyl acetate. The organic phase was then evaporated under vacuum in a Vortex Evaporator (Haake-Buchler, Sadle Brook NJ) and pellets were stored at -20°C. Before injection onto HPLC system, pellets were reconstitued with 200 µl of mobil phase buffer. The overall recovery for all compounds ranged from 65% to 75%.

For HPLC analysis, the mobil phases tested consisted of ternary mixtures of acetonitrile, methanol and phosphate buffer whose ratio organic phase/aqueous phase ranged from 45/55 to 60/40. The pH tested for each mobil phase was in the range of 2.5 to 7.0. Calibration curves for dmDNR , M I and M II were performed by injecting 10 to 100 µl of serial diluitions from the stock solutions; these curves were linear in the range of 10 ng/ml to 50 µg/ml with correlation coefficients always greater than 0.997.

The quantitation of dmDNR and its metabolites was achieved by regression analysis of the peak area ratio drug / internal standard.

RESULTS AND DISCUSSION

The goal of this work was to optimize an analytical method for a complete pharmacological and toxicological follow up of patients treated with the new DNR derivative 4-demethoxydaunorubicin. Because of the possible involvment of aglycone metabolites in the toxic side effects of anthracyclines (24,25), we developed an HPLC method for the contemporary detection of dmDNR , its 13-dihydroderivative and its more lipophylic aglycone metabolite in biological fluids. Critical points to our attention were a good separation of the drug from its metabolites, a low ratio area/height (A/H) of each peak, a reasonable time of analysis (in the range of 10 min) and sensitivity at nanograms level.

The characteristics of the mobil phases tested are summarized in Table 1.

From α to ε the eluent strength decreases along with the increase of the percent of aqueous phase. As a consequence, the total time of analysis increases from 6 min for the mobil phase α to more than 18 min for the mobil phase ε.

The influence of the different mobil phases on the peak shape (ratio A/H) of each compound is shown in Figure 2. When the aqueous phase was more than 50%, such as in mobil phase ε, the ratio A/H was always higher than 0.4, with unsatisfactory values of about 0.9 for M II. Increasing the eluent strength of the mobil phase, a progressive decrease of the ratio A/H was observed.

TABLE 1.

Composition of the Mobil Phases Tested.

Mobil Phase	CH_3CN (%)	CH_3OH (%)	Phosphate Buffer (%)
α	40	20	40
β	40	10	50
γ	35	15	50
δ	30	20	50
ε	30	15	55

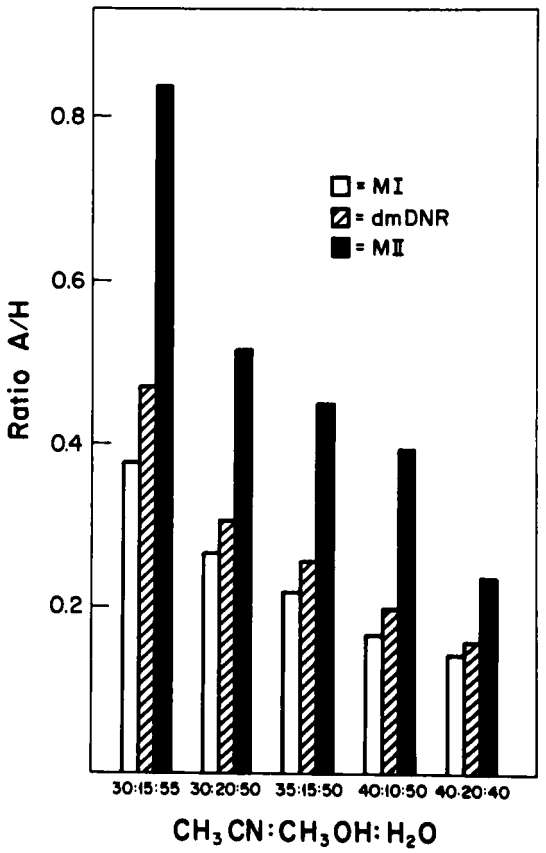

FIGURE 2. Relationship between the eluent strength and the peak Area/Height ratio.

The best result in terms of separation, peak shape and time of analysis was achieved with mobil phase B, consisting of acetonitrile:methanol:phosphate buffer 40:10:50. The optimal pH for this mobil phase resulted 4.7 $\pm$ 0.1. Under these conditions, at a costant flow rate of 2.0 ml/min, all compounds displayed ratios A/H < 0.5, and retention times were 2.2 , 3.2 , 4.2 and 9.2 min for the internal standard (K'= 0.7), M I (K'= 1.4), dmDNR (K'= 2.2) and M II (K'= 6.4), respectively (Figure 3). Limit of sensitivity of this assay (ratio peak to noise > 5) for dmDNR and its two metabolites was 20 ng/ml in plasma.

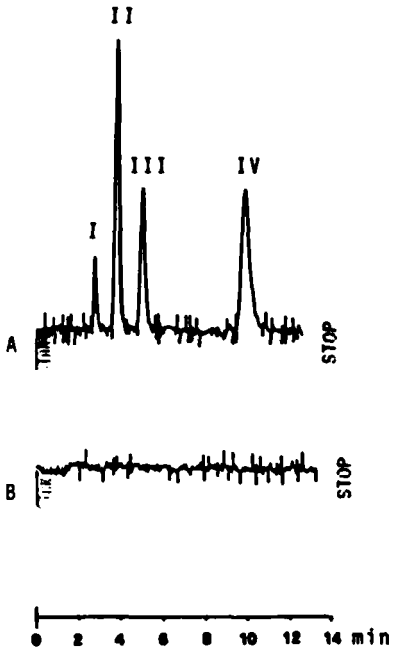

FIGURE 3. A) Chromatogram of extract from human plasma spiked with 500 ng/ml of DXR (I) M I (II) dmDNR (III) and M II (IV).
B) Chromatogram of extract from blank human plasma.

During these studies we noted that the peak of M II was unreasonably small in the chromatograms of the low M II concentrations (< 100 ng/ml) when the metabolite was incubated and extracted from whole blood instead of plasma. Furthermore, in the chromatograms of both low and high concentrations of M II appeared a peak at 5.3 min (K'= 3.1),identified by its chromatographic properties as the 13-dihydro-4-demethoxydaunorubicinone. Different concentrations of M II were therefore incubated for different periods of time with fresh human blood: the turnover of M II into its 13-dihydroderivative resulted to be function of time and inversely proportional to M II concentration. For example, when the M II concentration was 100 ng/ml, the conversion of M II in its 13-dihydroderivative was approximately 5% at 30 min and more than 80% after 24 hours, while for the higher concentration of 10 μg/ml, only 2.5% and 48% of M

II were converted into its 13-dihydroderivative after 30 min and 24 hours respectively. No significant loss of M II were detected after 24 hours incubation of the compound in plasma or water: the amount of the 13-dihydro-4-demethoxydaunorubicinone was always < 0.5%, that is the same amount detected in standard solutions as impurity. The phenomenon of M II reduction is likely to be attribuited to aldo-cheto reductases present in human erithrocytes (26).

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