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# QUANTITATIVE DETERMINATION OF LOW LEVELS OF DAUNOMYCIN AND DAUNOMYCINOL IN PLASMA BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

R. HULHOVEN* and J. P. DESAGER

*Laboratoire de Pharmacothérapie, Université Catholique de Louvain, 10 Minderbroedersstraat, B-3000 Leuven (Belgium)*

## SUMMARY

A high-performance liquid chromatographic method for the measurement of daunomycin and its main metabolite, daunomycinol, at low concentrations in plasma is described.

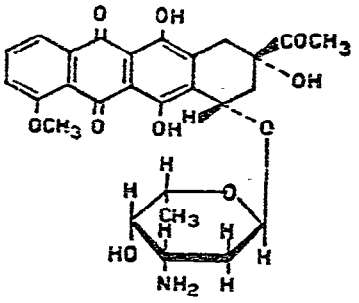
Quantitative determinations were obtained by the use of adriamycin, another cystostatic anthracycline antibiotic, as an internal standard. The separations were carried out on a 5-$\mu$m silica microsphere column with a quaternary solvent mixture as eluent. The components in the eluted peaks were detected by visible absorption at 490 nm and there were no interfering peaks. The advantages of the method are specificity, sensitivity, minimal pre-analysis sample work-up and small sample size. The method is sensitive for plasma levels of daunomycin and daunomycinol above 10 ng/ml.

Experimental animal data are presented to illustrate the application of the method.

## INTRODUCTION

Daunomycin (Rubidomycin or daunorubicin), a cytostatic antibiotic produced by *Streptomyces peucetius*, is used in acute leukaemias and malignant lymphomas. The aim of this work was to study the kinetics and metabolism of daunomycin in plasma, bile and urine by a rapid and reliable method. The quantitative determination of drugs and their metabolites in biological fluids is needed for correlating pharmacological activity. Various methods involving pre-purification have been described and are time consuming when operated so as to ensure the sensitivity and specificity required to give accurate data on a drug's metabolism. Total fluorescence of the biological extracts is very sensitive but not specific[1], whereas thin-layer chromatography followed by scraping off the fluorescent spots and fluorimetric dosage is a specific method[2] (Fig. 1).

* Aspirant du Fonds National de la Recherche Scientifique de Belgique.



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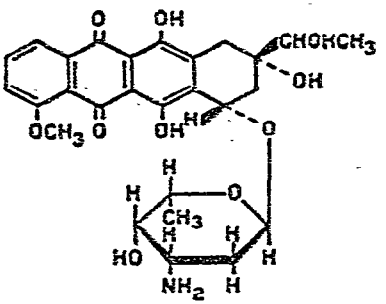
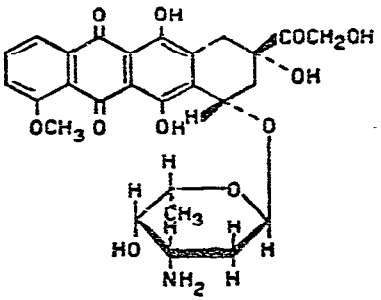


DAUNOMYCINOL



ADRIAMYCIN

Fig. 1. Structural formulae of daunomycin, daunomycinol and adriamycin.

Recently, high-performance liquid chromatography (HPLC) was introduced for the separation of adriamycin and its main metabolites in urine[3]. Quantitation has also been performed by a radioimmunoassay[4]. Considering the instability and very low volatility of daunomycin, HPLC was thought to be the optimal method, as for several other antibiotics (penicillins, cephalosporins, tetracyclines)[5].

A method of dosage of daunomycin in plasma by HPLC is described here. After a single extraction, in the presence of an internal standard (adriamycin), the drug and its reduced metabolite, daunomycinol, are determined simultaneously down to a level of 10 ng/ml.

## MATERIAL AND METHODS

### *Chemicals*

Pure samples of daunomycin and daunomycinol hydrochloride were obtained from Rhone-Poulenc-Specia (Paris, France). Adriamycin hydrochloride was provided by Farmitalia (Milan, Italy). The purity of these three samples was checked by thin-layer chromatography in various chromatographic systems. No aglycone moiety could be detected. Methanol, methylene chloride (Merck, Darmstadt, G.F.R.) and isopropanol (UCB, Brussels, Belgium) were of analytical grade and were not purified further.

Stock solutions of the three drugs were prepared as required by dissolving about 10 mg in 1 ml of water plus 9 ml of methanol and making up the volume to 100 ml with methylene chloride. A 1:100 dilution in methylene chloride–isopropanol (92:8, v/v) was used for daunomycin and daunomycinol. For the internal standard (adriamycin), 1 ml of the stock solution was diluted to 100 ml with pure methylene chloride, 20-ml aliquots of the diluted solution were evaporated to dryness using a Rotavapor and the residue was kept at −20° until required for use.

These procedures overcame problems linked to the instability of the molecules. The Millipore filters used with the 1225 sampling manifold were made of Mitex (10 μm) or Fluoropore (0.2 μm).

*High-performance liquid chromatography*

We used a DuPont series 848 pneumatic amplifier pump at 4500 p.s.i. with no pulse dampener. The detector was a DuPont series 837 spectrophotometer (200–600 nm) fixed at 490 nm with a sensitivity of 0.02 or 0.04 absorbance unit.

The system was equipped with a stainless-steel analytical column (250 × 2.1 mm) packed with Zorbax SIL, a totally porous silica microsphere packing of particle diameter 5 μm.

A six-port Rheodyne rotary injection valve fitted with a 200-μl loop was mounted between the pump and the column. All separations were carried out at room temperature.

The flow-rate of the mobile phase, methylene chloride–methanol–25% ammonia–water (90:9:0.1:0.8) was 0.9 ml/min. The retention times of daunomycin, adriamycin (internal standard) and daunomycinol were 2.5, 5 and 7 min, respectively, with this solvent system at 4500 p.s.i.

*Extraction procedure*

To a 12-ml stoppered tube were added successively 1 ml of plasma, 1 drop (0.04 ml) of 0.1 *N* sodium hydroxide solution, 1 ml of internal standard (adriamycin, 1.0 μg/ml) and 4 ml of extraction mixture (methylene chloride–isopropanol, 92:8).

After manual extraction for 1 min, the tube was centrifuged at 2300 *g* for 5 min. The lower layer was transfered on to a Millipore filter in order to remove suspended proteins and 2 ml of the extraction mixture were added to the residue. After a second extraction for 30 sec followed by centrifugation, the lower layer was transferred on to the same filter and filtered with a slight vacuum. The filter was rinsed with 1 ml of extraction mixture. The filtrate was evaporated to dryness in a 60-ml flask using a Rotavapor at 30°. Just before injection, the dry residue was solubilized with 1 ml of the elution mixture and 200 μl were injected rapidly. The syringe and loop were rinsed with elution solvent between injections.

*Quantitation*

A calibration graph for each compound in the range 0–3 μg/ml was obtained by assaying plasma containing known amounts of daunomycin and daunomycinol. By plotting the amount of drugs added against the ratio of peak height × retention time (in millimetres) (daunomycin *versus* internal standard and daunomycinol *versus* internal standard), Fig. 2 was obtained. The peak height × retention time ratio of daunomycin to internal standard was calculated for each sample and the amount of

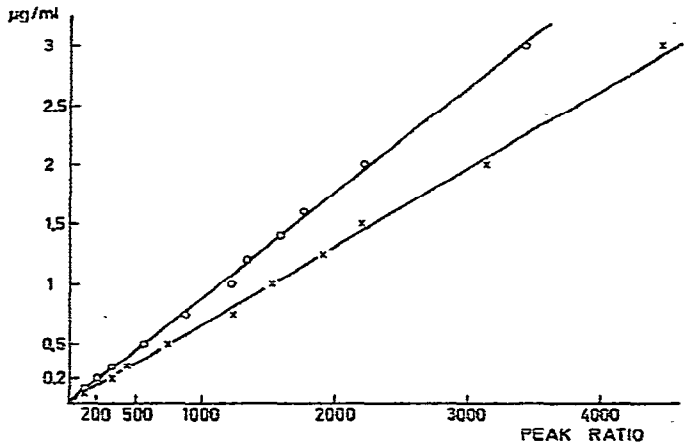



Fig. 2. Calibration graphs for daunomycin (○) and daunomycinol (×) in rabbit plasma at 490 nm.

$$\text{Peak ratio} = \frac{\text{peak height} \cdot \text{retention time (mm) of drug}}{\text{peak height} \cdot \text{retention time (mm) of internal standard}} \cdot 1000.$$

daunomycin determined by reference to the calibration graph. The same procedure was followed for daunomycinol.

The reproducibility and accuracy were found to be 3.5 ± 1% and 2 ± 0.5%, respectively. The sensitivity on an expanded scale was about 10 ng/ml and the recovery was 90 ± 3% for both drugs.

## RESULTS AND DISCUSSION

With UV detection at 235 and 254 nm, high peaks were obtained but they were disturbed by many unknown substances. With a view to increasing the selectivity, a spectrofluorometric detector was tested at 475 nm for excitation and 550 nm for detection; the selectivity was improved but not the sensitivity. However, using the spectrophotometric detector fixed at 490 nm, no pre-purification was needed, no interference was found and the selectivity was optimized. Typical chromatograms obtained with rabbit plasma are shown in Fig. 3.

The elution system proved to be suitable when used with the Zorbax SIL column, is demonstrated by the efficiency of 8000 theoretical plates per 10 cm for daunomycin at a pressure drop of 4500 p.s.i. and a flow-rate of 0.9 ml/min. The fraction of 25% ammonia in the mobile phase must be carefully controled in order to obtain the results shown in Fig. 3. The analysis is rapid and the peaks are sharp, contrary to the results obtained with adriamycin analyzed on a reversed-phase column with an elution gradient[3].

It proved to be sufficient to extract the sample twice and the addition of sodium hydroxide generated the free bases. Sample loss was corrected by using the internal standard. For quantitation, instead of the peak height ratio of daunomycin or daunomycinol to adriamycin, the retention time was taken into account for the calculation. This correction was introduced because we have observed slight variations in the retention times. Indeed, we did not work at equilibrium and the analysis was

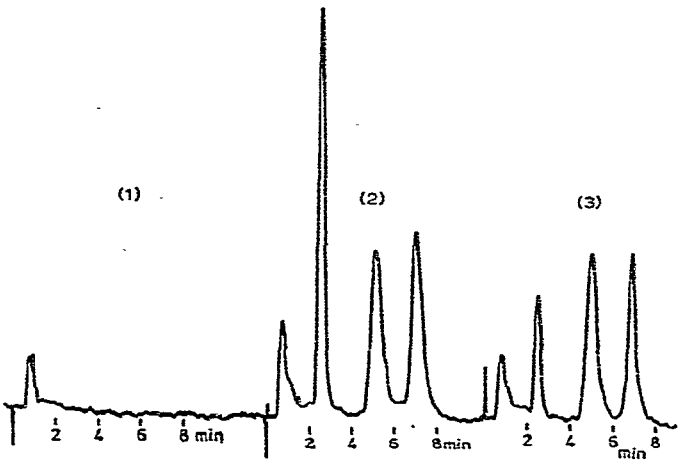



Fig. 3. Typical chromatograms of rabbit plasma at 490 nm: (1) blank; (2) 1 μg of daunomycin and 1 μg of daunomycinol added to a blank; (3) experimental sample containing 320 ng/ml of daunomycin and 920 ng/ml of daunomycinol.

performed within two or three hours. Also, some unknown and undetected substances must play the role of a stationary phase by partly coating the silica microspheres of the column, so increasing the retention times.

The 200-μl injection loop permits the direct detection of 10 ng/ml of each drug in plasma. For a kinetic study, this sensitivity is usually sufficient. Although radioimmunoassay[3,4] is more sensitive (0.15 pg/ml), it must be coupled with a separation method in order to overcome its lack of specificity towards drugs and metabolites.

The data obtained after a single intravenous injection of 5 mg/kg of daunomycin in four rabbits are reported in Table I. The values obtained are in the range of those reported elsewhere[2].

We intend to apply our method to the quantitative determination of adriamycin and reduced metabolite in biological fluids.

TABLE I

PLASMA LEVELS AFTER INTRAVENOUS INJECTION OF 5 mg/kg OF DAUNOMYCIN IN FOUR RABBITS

Results are mean values ± standard errors of the mean.

| *Time (min)* | *Daunomycin (ng/ml)* | *Daunomycinol (ng/ml)* |
|---|---|---|
| 5 | 405 ± 59 | 1.850 ± 310 |
| 15 | 243 ± 24 | 542 ± 54 |
| 30 | 141 ± 14 | 347 ± 47 |
| 60 | 75 ± 6 | 184 ± 15 |
| 120 | 36 ± 5 | 135 ± 10 |
| 180 | 32 ± 4 | 125 ± 9 |
| 240 | 24 ± 5 | 116 ± 6 |
| 300 | 24 ± 2 | 130 ± 12 |
| 360 | 21 ± 5 | 121 ± 27 |

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