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Daunorubicin metabolism: estimation of daunorubicin reductase

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Summary

1. Daunorubicin reductase, an enzyme ubiquitous in all mammalian tissues tested, converts daunorubicin, a cancer chemotherapeutic antibiotic, to daunorubicinol. A method for the estimation of daunorubicinol and daunorubicin reductase is described.

2. Daunorubicin and daunorubicinol were quantitatively extracted from enzyme assay mixtures with 0·3 N HCl in 50% ethanol and then completely hydrolysed to their respective aglycones. The aglycones were extracted, concentrated into toluene, separated by silicic acid microcolumn chromatography, and estimated by spectrofluorimetry.

3. Compared to previous methods, this method is highly reproducible and sensitive.

Introduction

Daunorubicin, currently one of the most active chemotherapeutic agents for inducing remissions in acute granulocytic leukaemia and in acute lymphocytic leukaemia (Bernard, Jacquillat, Boiron, Najean, Seligmann, Tanzer, Weil & Lortholary, 1967 ; Serpick & Wiernik, 1970) is metabolized extensively *in vitro* and *in vivo* by rat and man (Bachur & Cradock, 1970 ; Alberts, Bachur & Holtzman, 1971 ; Huffman, Bachur & Gee, 1970). A metabolic conversion common to these tissues is the reduction of daunorubicin (D1) to daunorubicinol (D2) by the enzyme

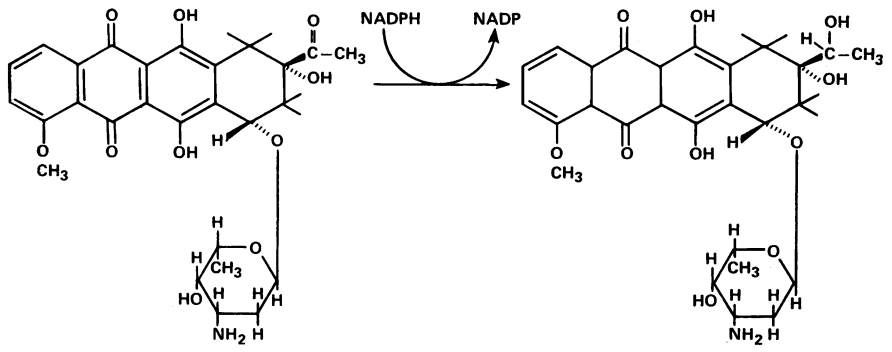

FIG. 1. Reaction catalysed by daunorubicin reductase.

daunorubicin reductase (Fig. 1) (Bachur & Gee, 1971). Since this conversion can occur in human blood cells (Huffman *et al.*, 1970) and since blood cells are the site of action of the drug in the leukaemias, the concentrations of daunorubicin reductase in these cells, as well as in other tissues, may bear a relationship to the pharmocodynamics of daunorubicin therapy.

Major problems in the study of the biochemical pharmacology of daunorubicin have been the tenacious binding of the drug and metabolites to tissue components, the instability of the glycoside linkage, and the physicochemical similarity of daunorubicin to the metabolite, daunorubicinol, which prevented an easy assay. In our earlier studies, the conversion of daunorubicin to daunorubicinol was estimated by extracting both substrate and product by an *n*-butanol extraction method, separating the product from the substrate by thin layer chromatography, eluting the product from the chromatograms and estimating the product by fluorescence assay. In addition, although NADPH was a cofactor in the daunorubicin reductase reaction, absorption at 340 nm by substrate levels of daunorubicin precluded the use of a spectrophotometric assay. A method is described which is based on conversion of substrate and product glycosides to aglycones with their quantitative extraction from the reaction media to an organic phase. Separation of the aglycones is accomplished by column chromatography. Measurement of the recovered aglycones is by fluorescence assay.

Methods

Daunorubicin HCl (Farmitalia) obtained from the Drug Development Branch, Cancer Chemotherapy National Service Center, NCI, NIH, was purified before use (Bachur & Cradock, 1970). Daunorubicinol was synthesized as previously described (Bachur, 1971). Silicic acid for column chromatography was prepared as described by Bachur, Masek, Melmon & Udenfriend (1965), and 250 μm silica gel G plates activated at 120° C for 1 h were used for thin layer chromatography (Bachur & Cradock, 1970).

Male, 200 g Sprague-Dawley rats from NIH stock and fed and watered *ad libitum* were the source of tissues. Animals were killed by a blow to the neck, the tissues excised, washed in ice cold saline and homogenized in two volumes (w/v) of Tris Cl buffer, pH 7·4, 0·05 M with a Potter Elvehjem type teflon and glass tissue grinder. This homogenate was used as enzyme source.

Human blood cells were prepared from freshly drawn heparinized venous blood which was centrifuged at 1,500 *g* for 10 minutes. The cells were mixed with two volumes (w/v) of Tris Cl buffer, pH 7·4, 0·05 M and homogenized as above.

The enzymatic conversion of D1 to D2 was carried out in a total volume of 0·5 ml containing 50 μmol Tris Cl, pH 7·4, 1 μmol NADPH, 0·582 μmol daunorubicin, and the tissue homogenate or extract. Generally, 0·1–0·5 mg protein of liver or kidney homogenate was sufficient to convert 10% of the D1 to D2. The reaction mixture was incubated at 37° C with shaking for 30 minutes. For the accurate estimate of daunorubicin reductase, the amount of tissue extract added should be adjusted to convert less than 10% of the substrate.

The estimation of D2 production by *n*-butanol extraction, thin layer chromatography, and fluorescence analysis was reported previously (Bachur & Gee, 1971). With the new method, 2·0 ml of 0·3 N HCl in 50% ethanol is added to the reaction

vessels to stop and extract the reaction. After centrifugation, the clarified supernatant solutions are transferred to tubes which are placed in a boiling water bath for 15 minutes. The tubes are cooled and 7·5 ml of water is added followed by 2·0 ml of toluene. The phases are thoroughly mixed for at least 2 minutes. On separation of the phases by centrifugation, all of the red colour of daunorubicin derivatives should be in the upper toluene phase. A 0·2 ml aliquot of the toluene phase is applied to a 3 × 40 mm column of silicic acid in toluene. About 60 ml of a toluene methanol mixture (167:1 v/v) is used to wash the substrate completely from the silicic acid while the enzymatic product remains bound. Preceding the elution of hydrolysed substrate, a small (<1%) amount of red fluorescent material elutes from the column. This product is seen in all samples and is not related to the enzymatic reaction. Enzymatic product derivative is eluted from the column completely with 2·0 ml of absolute methanol and collected. The volume of the methanol eluate is adjusted with methanol and the fluorescence at 585 nm in determined with activation of 470 nm. Completion of the separation can be monitored by thin layer chromatography (Bachur & Cradock, 1970).

Results

Comparative fluorescence of daunorubicin and daunorubicin aglycone

Daunorubicin and daunorubicinol were hydrolysed in 0·3 N HCl–50% ethanol by heating for 15 min at 100° C. The solvents were evaporated to dryness and the solutions were reconstituted to 5 ml with methanol, then compared to equimolar solutions of unhydrolysed D1 and D2. There were no differences in fluorescence or absorption of the glycosides or the aglycones.

Hydrolysis timing

Hydrolysis of daunorubicin or daunorubicinol in an acid alcohol solution was completed by 5 min at 100° C (Fig. 2). Extended heating to 30 min had no effect on the total recovery of fluorescence indicating stability of both daunorubicin agly-

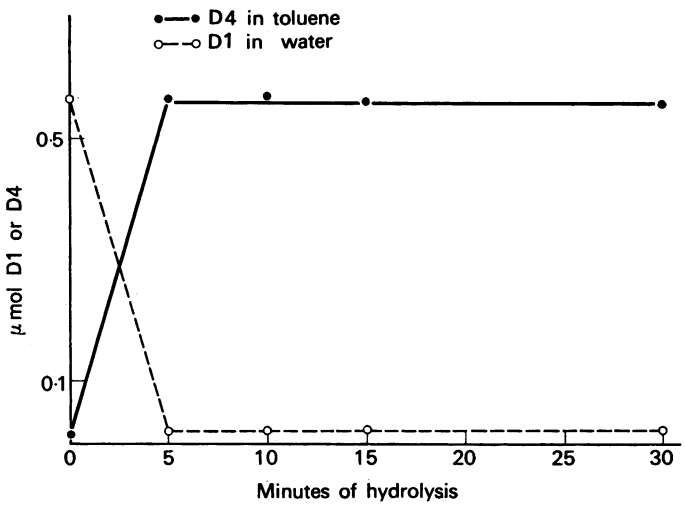

FIG. 2. Conversion of daunorubicin (D1) to daunorubicin aglycone (D4) by acid hydrolysis at 100° C.

cone (D4) or daunorubicinol aglycone (D3) under these conditions. After hydrolysis and dilution of the methanol concentration, the aglycones could be quantitatively extracted into toluene.

Solvent effects on fluorescence of aglycones

The effect of solvent on the quantum efficiency of daunorubicin fluorescence is shown in Fig. 3. Since the fluorescence is reduced in toluene, volume adjustments of the eluted product are made with methanol and the relationship of toluene to methanol concentration is kept constant by keeping the column size constant.

Comparison with other methods

When comparable enzymatic reactions are extracted by the *n*-butanol or the acid alcohol method, the level of total fluorescence recovery is found to average 68·8% for *n*-butanol and 96·3% for the acid alcohol (Table 1). These results are similar to those reported previously (Bachur, Moore, Bernstein & Liu, 1970). Further processing of the extraction mixtures and assay of metabolite by both techniques as described in **Methods**, indicated the level of conversion was 47% higher with the liver, 21% higher with kidney, and 21% higher with human blood cells in the acid alcohol column assay than in the *n*-butanol TLC assay.

Repeated daunorubicin reductase determinations of blood cell and kidney homogenates indicated a high degree of reproducibility for the extraction technique as

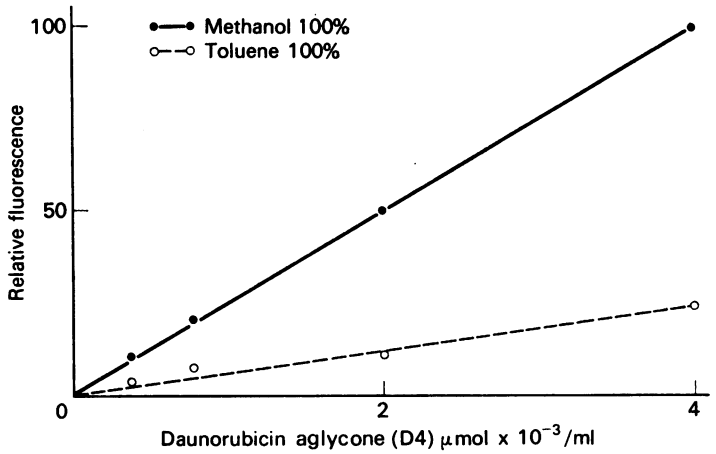

FIG. 3. Effect of solvent on the relative fluorescence of daunorubicin aglycone (D4). Activation 470 nm, emission 585 nm.

TABLE 1. *Tissue concentrations of daunorubicin reductase by different techniques*

Homogenate	*n*-Butanol extraction+TLC recovery %	nmol D2/mg protein	Acid alcohol extraction+column recovery %	nmol D2/mg protein
Rat liver	70·4	11·6	93	17·0
Rat kidney	65	32·8	98	39·7
Human blood cells	71	1·52	98	1·84

Reaction details and methods for determining daunorubicinol (D2) are described in **Methods.** Recovery represents total fluorescence extracted as compared to total fluorescence added. The figures represent the average of duplicate experiments.

well as linearity in the assay (Fig. 4). About 20% conversion of daunorubicin to daunorubicinol occurred before the reaction slowed.

Identity of fluorescent species

When portions of the toluene extracts from reaction mixtures were chromatographed by silicic acid thin layers, the only significant fluorescent materials seen were the aglycones of daunorubicin and daunorubicinol.

Discussion

The conversion of daunorubicin to daunorubicinol by solid tissues as well as by human blood components is critical to the evaluation of the pharmacodynamics and mechanism of action of the chemotherapeutic agent.

The major advantages of this method of assay are as follows. (1) This is a direct measurement of the product. (2) The use of acid-alcohol results in better extraction of the daunorubicin and daunorubicinol from cell components resulting in higher efficiency and reproducibility. (3) A means of concentrating the diluted sample after extraction increases the sensitivity of the method. (4) Higher substrate concentrations can be used in this method and there is less chance of contaminating the product with substrate as occurs in the *n*-butanol TLC method. (5) No product loss is associated with the microcolumn technique for separating substrate derivative from product derivative. (6) The range of linearity for the fluorescence assay of daunorubicin ($4{\cdot}3 \times 10^{-4}$ to 17·2 nmol/ml) (Bachur *et al.*, 1970) allows for the estimation of a wide range of enzyme activity.

A problem of the acid-alcohol microcolumn method involves the solvent effects on fluorescent yield. Care must be taken to maintain an unvarying concentration ratio of methanol and toluene since fluorescent yield of the drug and the metabolite will vary depending on the methanol concentration of the solvent.

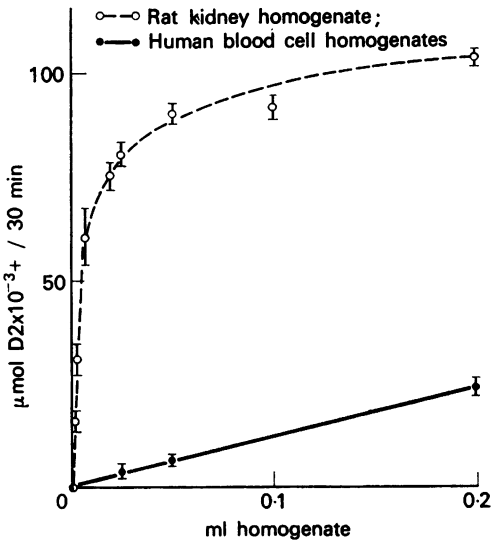

FIG. 4. Enzymatic formation of daunorubicinol; effect of enzyme concentration. The details of the reaction mixture are in **Methods.** Each point represents the average of four determinations with the standard error of mean. The protein concentration for rat kidney homogenate was 22·3 mg/ml and for human blood cell homogenate was 58·0 mg/ml.

Since daunorubicinol has cytotoxic activity (Bachur & Adamson, unpublished observations), and can inhibit both DNA and RNA metabolism in L1210 mouse leukaemia cells (Meriwether, Bachur & Gee, 1971), the concentration of daunorubicin reductase may be of importance in understanding the pharmacodynamics of daunorubicin. There are indications that the *in vivo* production of daunorubicinol effects the pharmacodynamics of the drug in animals and in humans (Huffman *et al.*, 1970).

REFERENCES

ALBERTS, D., BACHUR, N. R. & HOLTZMAN, J. L. (1971). The pharmacokinetics of daunomycin in man. *Clin. Pharmac. Ther.*, **12**, 96–104.

BACHUR, N. R. (1971). Daunorubicinol, a major metabolite of daunorubicin: Isolation from human urine and enzymatic reactions. *J. Pharmac. exp. Ther.*, **177**, 573–578.

BACHUR, N. R. & CRADOCK, J. (1970). Daunomycin metabolism in rat tissue slices. *J. Pharmac. exp. Ther.*, **175**, 331–337.

BACHUR, N. R. & GEE, M. (1971). Daunorubicin metabolism by rat tissue preparations. *J. Pharmac. exp. Ther.*, **177**, 567–572.

BACHUR, N. R., MASEK, K., MELMON, K. L. & UDENFRIEND, S. (1965). Fatty acid amides of ethanolamine in mammalian tissues. *J. biol. Chem.*, **240**, 1018–1024.

BACHUR, N. R., MOORE, A. L., BERNSTEIN, J. G. & LIU, A. (1970). Tissue distribution and disposition of daunomycin (NSC-82151) in mice: fluorometric and isotopic methods. *Cancer Chemoth. Reports*, **54**, 89–94.

BERNARD, J., JACQUILLAT, C., BOIRON, M., NAJEAN, J., SELIGMANN, Y., TANZER, J., WEIL, M. & LORTHOLARY, P. (1967). Essai de traitment des leucemies aigues lymphoblastique et myeloblastiques par un antibiotic nouveau: la rubidomycine etude de 61 observations. *Presse med.*, **75**, 951–956.

HUFFMAN, D. H. BACHUR, N. R. & GEE, M. (1970). Hematological metabolism of daunomycin in man. *Clin. Res.*, **18**, 472.

MERIWETHER, W. D. BACHUR, N. R. & GEE, M. (1971). Inhibition of DNA and RNA metabolism by daunorubicin (D1) and its major metabolite (D2) in L1210 mouse leukemia. *Clin. Res.*, **19**, 494.

SERPICK, A. A. & WIERNIK, P. H. (1970). Remission induction in adult acute nonlymphocytic leukemia: A comparison of daunomycin with combination therapy. *Proc. 10th Int. Congr. Cancer*, Houston, Texas.

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