Journal of Chromatography, 221 (1980) 139—144
Biomedical Applications
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROMBIO. 665

EXTRACTION AND QUANTIFICATION OF DAUNOMYCIN AND DOXORUBICIN IN TISSUES

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(First received April 10th, 1980; revised manuscript received June 24th, 1980)

SUMMARY

The measurement of intracellular concentrations of the anti-cancer drug doxorubicin was performed by the application of a simple cell extraction technique combined with a rapid high-performance liquid chromatographic separation. Quantitation was done by fluorescence detection. The extraction procedure was non-degradative and the mean recovery of drug was 95%. A high drug extraction efficiency was confirmed with radiolabeled [^{3}H]doxorubicin. The method is applicable to normal and neoplastic tissue.

INTRODUCTION

Quantitation of the tissue content of two important anti-cancer agents, doxorubicin and daunomycin, has been difficult. Assays have been carried out using either demanding thin-layer chromatography techniques [1] or fluorometric measurements that are unable to resolve metabolites of the drugs [2].

Measurements of cell levels of these drugs are of potential therapeutic importance, since resistance to these agents has been shown to be related to alterations in drug transport [3—5] in several experimental tumors. Whether or not changes in drug transport are important in drug resistance to these agents in human tumors is yet to be demonstrated.

Recently, high-performance liquid chromatographic (HPLC) techniques

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have been described for analyzing daunomycin and doxorubicin in urine and plasma [6–8]. The present study describes a rapid tissue extraction method modified from Israel et al. [7] coupled with a simple HPLC separation that can be used to determine cellular drug levels of daunomycin or doxorubicin. The extraction efficiency is high and is confirmed by measurement with radiolabeled doxorubicin.

EXPERIMENTAL

Reagents

Doxorubicin was obtained from commercial sources (Adria Laboratories, Dublin, OH, U.S.A.). Radiolabeled doxorubicin ([^{3}H]doxorubicin) was a gift of New England Nuclear (Boston, MA, U.S.A.), specific activity 0.5 Ci/mmol. The purification of the labeled material is described below. Doxorubicinol and doxorubicinone were prepared from doxorubicin [9]. Daunomycin was obtained from the National Cancer Institute (Bethesda, MD, U.S.A.). Methanol (distilled in glass) was obtained from Burdick & Jackson Labs., Muskegon, MI, U.S.A. R.P.M.I. 1640 was obtained from Associated Biomedic Systems, Buffalo, NY, U.S.A.

Tissues

The cells used in experiments were either peripheral blood (PB) mononuclear cells obtained from heparin anticoagulated venous blood drawn from normal volunteers and then separated on Ficoll-Hypaque gradient (PB lymphocytes) or HL-60 cells grown from a stock kindly provided by R. Gallo (National Cancer Institute). HL-60 is a recently described cell line derived from a patient with acute progranulocytic leukemia [10]. HL-60 cells were grown in R.P.M.I. 1640 medium supplemented with 20% fetal calf serum, 1 μM glutamine, and 100 units penicillin per ml and 50 μg streptomycin per ml in Corning T-30 flasks in 95% air, 5% carbon dioxide.

Cell incubations

Cells were suspended at 2×10^6/ml in sterile Dulbecco's phosphate buffered saline (PBS), pH 7.4, at 37°C with drug concentrations and incubation times as specified. Incubations were in screw-capped glass tubes in air. Aliquots of HPLC purified, ^{3}H-labeled doxorubicin (1.85×10^4 disintegrations per second/10^6 cells) were added to incubation mixtures. Tubes were protected from light during the incubations.

Drug extractions

Samples of cells were extracted following incubations. Cells were centrifuged at 225 *g* for 5 min. The supernatant incubation medium was removed and cells were washed twice in 0.5 ml PBS. The incubation medium and all the washes for each sample were combined and subsequently handled for extraction in the same manner as the cell pellet. The washed cell pellet was resuspended in 1 ml PBS. One extract was prepared from the resuspended cell pellet and another from the pooled washes and incubation medium for each sample. These extracts were obtained by adjusting the pH with 200 μl Tris

buffer, pH 8.4, then extracting twice with four volumes of chloroform—methanol (9:1). The organic phase was removed, evaporated on a Buchi Rotavapor (Brinkmann Instruments, Westbury, NY, U.S.A.) over a 40°C water bath and the residue redissolved in the HPLC mobile phase for injection as described below. All steps in the extraction procedure were conducted in reduced room light. Samples redissolved in mobile phase were stable when stored at —20°C for at least 1 week.

High-performance liquid chromatography

The chromatographic separations were accomplished on a DuPont Model 850 liquid chromatograph (DuPont, Wilmington, DE, U.S.A.) equipped with a 30 cm × 3.9 mm I.D. μBondapak C_{18} column of 10 μm particle size (Waters Assoc. Waltham, MA, U.S.A.). An isocratic solvent system (mobile phase) was used consisting of 0.05 *M* NaH_2PO_4—methanol (35:65, v/v). Solvent was degassed by continuous helium sparging. The flow-rate was 1.5 ml/min and the column oven temperature was 36°C.

Ultraviolet absorption was monitored with a DuPont Model 850 fixed wavelength absorbance detector set at 254 nm. Fluorescence was measured with a Model SF-970 liquid chromatography fluorometer (Schoeffel Instrument, Westwood, NJ, U.S.A.). The excitation wavelength was 482 nm (tungsten lamp) and the emission wavelength was 580 nm (Schoeffel filter No. 2-73, low cut-off at 550 nm).

Triangulation of the peak area (UV or fluorescent detector) was used for quantitation. The standard curve was linear to at least 100 ng (185 pmol) with a correlation coefficient of 0.9998. The lower limit of sensitivity for accurate quantification was 5 ng per injection.

Radioactivity was measured using 10 ml/vial of Beckman Ready Solv HP scintillator in a Beckman LS150 liquid scintillation spectrometer. The ^3H counting efficiency was 30—40%. Counts were corrected by reference to a quench curve derived from external standard ratio measurements.

The [^3H]doxorubicin supplied by New England Nuclear was purified by collection of fractions corresponding to the fluorescent peak on HPLC injection. On re-injection the material was determined to be 94% pure. The [^3H]doxorubicin required periodic (approximately every 2 weeks) purification before experimental use.

RESULTS

HPLC separation

Injection of a mixture of daunomycin, doxorubicin, and two compounds reported to be metabolites of doxorubicin, namely doxorubicinol and doxorubicinone, showed excellent resolution of native drug from metabolites (Fig. 1). The compounds were recovered in the sequence doxorubicinol, doxorubicin, doxorubicinone, and daunomycin with retention times of 3.4, 3.9, 4.7, and 5.6 min, respectively.

Tissue extraction studies

In studies employing cell incubation with drug, the cell pellets were extracted and the washes were pooled with the incubation medium for

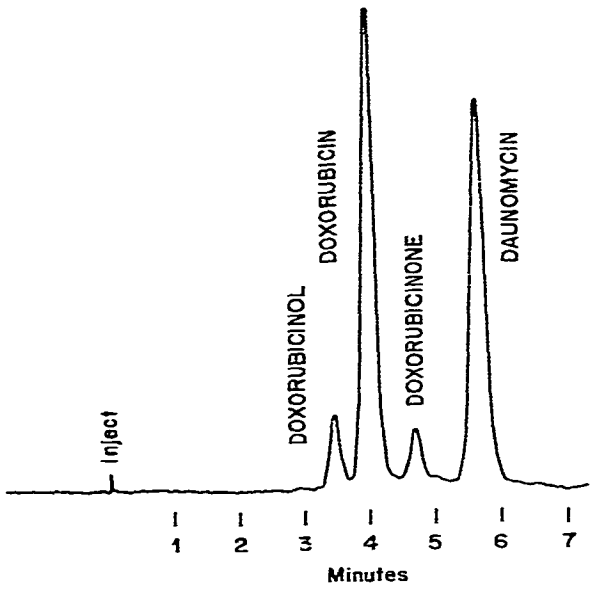

Fig. 1. HPLC separation of doxorubicin and daunomycin from related compounds using fluorescence detector. This represented the injection of an aliquot of a 1:6:1:6 mixture of doxorubicin, two metabolites of doxorubicin (doxorubicinol and doxorubicinone) and daunomycin with the solvent conditions as described under Experimental.

extraction separate from the cells. Aliquots of these extracts were injected into the liquid chromatograph. The drug content of the samples was determined both by triangulation of area of the fluorescence peak compared to the standard curve and by scintillation counting of the HPLC effluent fraction corresponding to the observed fluorescent peak. The lower limit of recognition was approximately at the level of 2 ng per sample; the limit for precise quantification was 5 ng per injection.

As shown in Table I, the cellular content of drug increased as the incubation time increased from 1 to 2 h with normal peripheral blood mononuclear cells. In addition, the cellular content of drug increased in a linear manner (Fig. 2)

TABLE I

DOXORUBICIN RECOVERY FOLLOWING INCUBATION WITH 10^6 PB LYMPOCYTES IN 0.5 ml PBS WITH 142 ng [^{3}H]DOXORUBICIN

Mean of 4 representative experiments.

	Drug in cells (ng)		Drug in media and washes (ng)		Total drug			
	fluorescence	^{3}H	fluorescence	^{3}H	fluorescence		^{3}H	
					ng	%	ng	%
1 h	33.75	37.6	124.75	132.1	158.5	112	169.7	119
2 h	48.32	49.1	87.0	85.5	135.32	96	134.6	94.7

as the drug concentration was increased during incubation in HL-60 cells (Table II).

The close agreement in both sets of experiments of drug assay by HPLC fluorescence measurement and by ^{3}H-label scintillation counting supported the observation that the extraction procedure did not result in loss of ^{3}H label. The mean total drug recovery was 95% in these experiments. The efficiency of total drug recovery in these experiments indicates that but little of the cellular content of drug might have been unextractable. Hence, the use of an internal standard, such as [^{3}H]doxorubicin added prior to the extraction, can be justified.

Careful analysis of the HPLC patterns in repeated runs and the recovery data revealed no significant evidence of drug metabolite formation with these cell types under these incubation conditions.

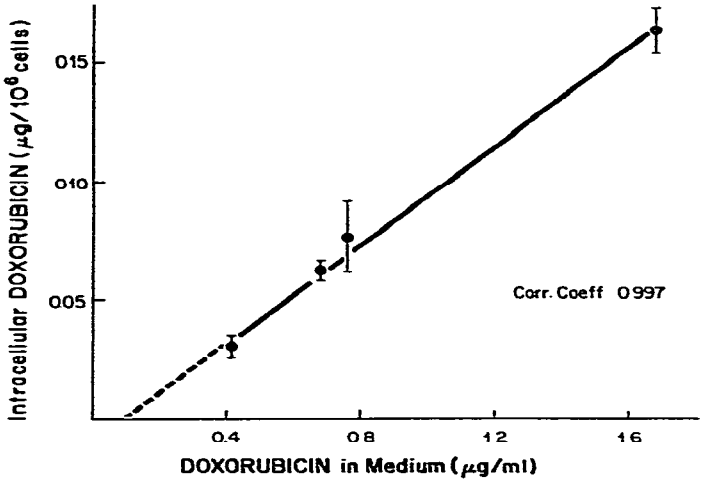

Fig. 2. Intracellular doxorubicin concentration in HL-60 cells. The recovery of intracellular doxorubicin following 1-h incubation of HL-60 cells with increasing concentrations of the drug in the incubation medium.

TABLE II

DOXORUBICIN RECOVERY FOLLOWING INCUBATION WITH HL-60 IN 0.5 ml PBS WITH INCREASING QUANTITIES OF [^{3}H]DOXORUBICIN FOR 1 h AT 37°C

Mean of 4 representative experiments.

Drug added (ng)	Cellular drug content (ng)		Drug in medium and washes (ng)		Total drug recovered			
	Measured by fluorescence	Measured by radiolabeling	fluorescence	^{3}H	Measured by fluorescence		Measured by radiolabeling	
					ng	%	ng	%
206	30	28	142.0	164.2	172.0	83	192.2	93
340	67.3	57.1	256.7	259.6	323.0	95	316.7	93
382	76	75.1	298.3	285.5	374.3	97.9	360.6	94.3
843	160.2	165.3	593.3	605.2	753.3	89.3	770.5	91.3

DISCUSSION

In the therapy of cancer in man the most effective drugs currently available are doxorubicin and daunomycin. Techniques providing accurate measurement of intracellular doxorubicin content and capable of detecting metabolites are therefore potentially important in exploring mechanisms of drug resistance in human tumors and for understanding the effect of agents that may modify the toxicity of doxorubicin.

The analytic procedures were modified from those of Israel et al. [7], who studied drug content in biological fluids. Their systems were not applicable to tissues, because they resulted in drug degradation and poor recovery. These problems led to the present extraction and analytic steps.

The present study shows that using highly purified ^{3}H-labeled doxorubicin we can confirm the efficient, non-degradative extraction of doxorubicin from cells and its reliable measurement in a HPLC system.

The results with HL-60 cells and peripheral blood lymphocytes show that, under these incubation conditions, cells rapidly concentrate doxorubicin. Brief incubation of HL-60 cells in doxorubicin in the range of drug concentrations used does result in inhibition of growth. Therefore, this technique provides the opportunity to determine cellular drug concentrations for correlative analysis with target cell cytotoxicity.

ACKNOWLEDGEMENTS

This work was supported by NIH Grants 18132 and 23115, the Heddens-Good Foundation, and the McDermott Foundation.

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