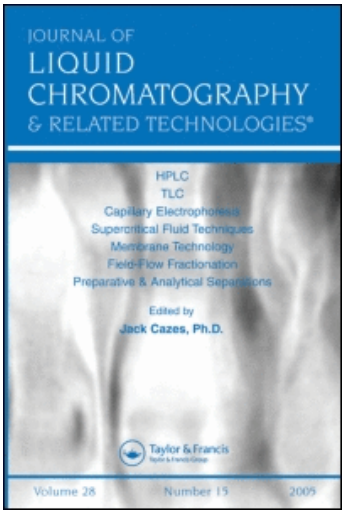

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H. J. Issaq[a]; N. H. Risser[a]; A. Aszalos[b]
[a] Chemical Carcinogenesis Program, NCI Frederick Cancer Research Center, Frederick, Maryland [b] Chemotherapy Fermentation Laboratory, NCI Frederick Cancer Research Center, Frederick, Maryland

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THIN-LAYER CHROMATOGRAPHIC SEPARATION AND QUANTITATION OF THE ANTI-TUMOR AGENT DAUNORUBICIN IN FERMENTATION MEDIA*

H.J. Issaq[1], N.H. Risser
Chemical Carcinogenesis Program

and

A. Aszalos
Chemotherapy Fermentation Laboratory

NCI Frederick Cancer Research Center
Frederick, Maryland 21701

INTRODUCTION

Daunorubicin (1,2), an antibiotic produced by the microorganism Streptomyces penceticus, can be used in the treatment of both acute leukemia and solid tumors in human (3). Extensive clinical trails of this compound are being conducted by the National Cancer Institute. Production of daunorubicin by fermentation and its isolation from fermentation broth has been described elsewhere (4). In the course of producing sufficient daunorubicin for clinical trails, the Chemotherapy Fermentation Laboratory

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[1]Author to whom correspondence should be addressed.

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of the Frederick Cancer Research Center required a fast quantitative assay for daunorubicin in fermentation broth. This paper describes a one-dimensional thin-layer chromatographic system for the separation of daunorubicin and its in situ quantitation by densitometry.

METHODS

Apparatus

Standard TLC glass tanks were used for plate development. Quantitative densitometric measurements were made (at 480 nm) on a Schoeffel SD 3000 spectrodensitometer connected to a Hewlett-Packard 3352A laboratory data system through an A/D converter.

Reagents

All solvents used were glass distilled (Burdick and Jackson, Muskegon, MI), or of Fisher certified ACS grade. Daunorubicin was supplied by Dr. J. Douros (NCI) and used without further purification; TLC of the compound in chloroform:methanol:acetic acid (65:10:10) on silica gel plates (EM silica gel 60) showed only one spot (R_f 0.37). Drummond micropipettes were used for applying the samples.

Preparation of Standard

100 µg daunorubicin were dissolved in 5 ml of acetone:deionized water (1:1 v/v).

Preparation of the Sample

Several different glycosides of daunorubicin are produced during fermentation. Most of these can be converted to daunorubicin by mild acid hydrolysis. The mixture is hydrolysed before application of the sample to the TLC plates. A 10 milliliter representative sample of the fermentation broth is transferred to a 25 ml Erlenmayer flask and 300 mg of oxalic acid added, after which the flask is placed in a 50°C waterbath for 45 minutes. The flask is then removed, and the sample cooled down to 10-15°C. A 1 ml aliquot of the daunorubicin oxalate solution is withdrawn from the top of the flask, and

mixed with 1 ml acetone in a test tube, after which 2-5 μl are spotted on the TLC plate.

TLC Development and Detection

Standard daunorubicin solution and broth media (2 μl) were applied side-by-side on a 20 cm TLC plate, and development was carried out with chloroform: methanol:acetic acid (65:10:10). After development, the plate was dried in a current of cold air and the density of the spots measured.

RESULTS AND DISCUSSION

Daunorubicin is separated from other components of the media by developing the TLC plate in chloroform:methanol:acetic acid (65:10:10). Under long-UV light the spots shown in Table I were observed. Note that daunorubicin is well resolved from other impurities which makes quantitative assay very easy. Drying the spots after spotting the samples on the plate affected the separation of daunorubicin contained in the media but not from the standard solution. The optimum drying time is 1 minute with a hair dryer. Also, it is important to spot a minimum amount of sample, 2 to 6 microliters. To aid

TABLE I R_f values and color of spots obtained when fermentation broth containing daunorubicin was spotted on silica gel plates and developed in chloroform:methanol:acetic acid (65:10:10).

R_f x 100	Color
0	Blue
9	Purple
19	Green
37	Daunorubicin
54	Faint Red
96	Faint Red

the drying of the spots, since the sample is prepared in water, acetone was added (1:1 v/v) before spotting.

The recovery of daunorubicin from fermentation media was tested by spiking two broth samples, with 25 mg/ml daunorubicin. Extraction was carried out as described in the experimental section. 2 and 3 µl of the extract were spotted on the TLC plate, developed and the density of daunorubicin was measured. The results indicate better than 90% recovery (Table II). At the time of this investigation, the method used for assaying daunorubicin in fermentation broth required several extraction and quantitation by high performance liquid chromatography, HPLC (5). The TLC method described here is faster, easier to perform and assays 15 samples per 20 x 20 cm plate simultaneously. Also, when the TLC method was compared to the old HPLC method by assaying over 60 broth samples, a very good agreement was found between both methods. A sample of these results is given (Table III). Quantitative densitometric measurements of daunorubicin in 1:1 v/v acetone: water and in fermentation broth gave a linear calibration curve (Figure 1). It is worth noting that this method is accurate for assaying in the range of 10-100 µg/ml daunorubicin.

TABLE II Recovery of daunorubicin from fermentation broth.

Trial	Sample #	Volume Spotted µl	Expected µg	Found µg	Recovery %
1	1	2	50	48	96
1	1	3	75	68	91
1	2	2	50	45	90
1	2	3	75	71	95
2	1	2	50	46	92
2	1	3	75	63	84
2	2	2	50	48	96
2	2	3	75	70	93

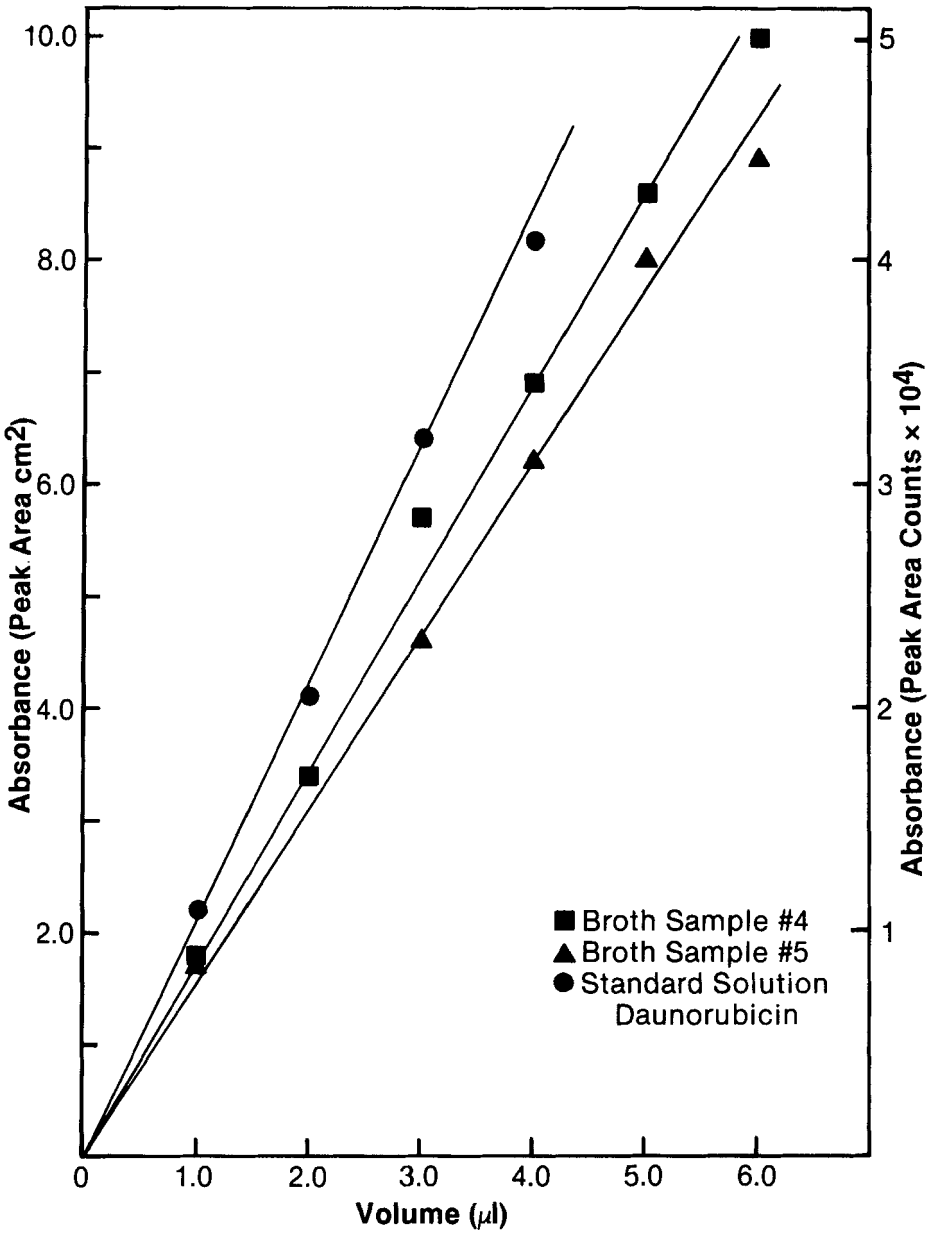

FIGURE 1 Calibration curves for standard solution of daunorubicin, and for daunorubicin in broth samples.

TABLE III Comparison of TLC and HPLC quantitative data for daunorubicin in fermentation broth.

Sample #	TLC (μg/ml)	HPLC (μg/ml
1	25.7	28.0
2	41.9	42.0
3	34.2	34.6
4	15.5	14.0
5	57.5	53.0
6	43.8	43.0
7	41.5	41.6
8	21.5	20.5
9	30.0	29.0
10	94.4	93.3

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