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## Note

# Thin-layer chromatographic analysis of adriamycinone in fermentation broths

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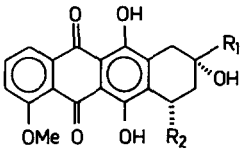
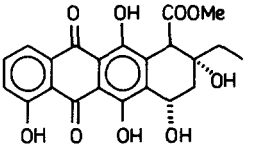
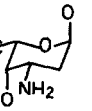
The anthracycline antibiotics, in particular daunomycin (daunorubicin) and its hydroxy analogue adriamycin (doxorubicin) are important antitumour agents[1,2]. As these anthracycline antibiotics were originally produced in small concentrations by *Streptomycetes* cultures, extensive work has been carried out to improve the fermentation and the strain productivity[3]. During these studies, a rapid and easy method was needed for qualitative and quantitative analysis of adriamycin and related compounds in fermentation broths. The separation and determination of anthracyclines in biological fluids have been achieved with thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) with fluorescence detection[4–6]. In fermentation broths, TLC has been applied only for qualitative determinations of anthracyclines and anthracyclinones[7,8]. We report here a simple and rapid semi-quantitative TLC method for adriamycin via adriamycinone in fermentation broths as a screening method without the need for special instrumentation. The method





(8)

| | | |
|---|---|---|
| (1) Daunomycin | $R_1 = COCH_3$ | $R_2$=  |
| (2) Adriamycin | $R_1 = COCH_2OH$ | |
| (3) 13-Dihydrodaunomycin | $R_1 = CHOHCH_3$ | $R_2$ = HO |
| (4) Daunomycinone | $R_1 = COCH_3$ | $R_2$ = OH |
| (5) Adriamycinone | $R_1 = COCH_2OH$ | $R_2$ = OH |
| (6) 13-Dihydrodaunomycinone | $R_1 = CHOHCH_3$ | $R_2$ = OH |
| (7) 7-Deoxy-13-dihydro-daunomycinone | $R_1 = CHOHCH_3$ | $R_2$ = H |
| (8) ε-Rhodomycinone | | |

consists of a hydrolysis step, solvent extraction of the anthracyclinones from the fermentation broths, development on a silica gel TLC sheet, silica gel–solvent transfer of the separated adriamycinone, and quantitation by fluorimetric assay.

## MATERIALS AND METHODS

### *Chemicals*

The anthracyclinones ε-rhodomycinone, adriamycinone, daunomycinone, 13-dihydrodaunomycinone, 7-deoxy-13-dihydrodaunomycinone were prepared and isolated in our laboratories. Their identities were confirmed by electron-impact mass spectrometry and protonnuclear magnetic resonance spectroscopy. The standard samples of adriamycin and daunomycin were provided by Dr. Grein from Farmitalia, Milan, Italy. All solvents were distilled before use.

### *Fermentation*

The antibiotic adriamycin was produced by the mutant strain F 23/II-8 of *Streptomyces peucetius* var. *caesius*, which was maintained in the strain collection of the Central Institute of Microbiology and Experimental Therapy, Jena.

All fermentations were conducted under submerged culture conditions according to the usual two-stage process in 500-ml and 20-l flasks, respectively. Seed cultures were prepared in a medium containing glucose (1.5%), soya meal (1.5%), calcium carbonate (0.1%) sodium chloride (0.5%), potassium dihydrogen phosphate (0.1%) and incubated at 28–30°C for 48 h. These seed cultures were used to inoculate (5%) a fermentation medium composed of glucose (6%) dried bakers yeast (3%), sodium chloride (0.2%), potassium dihydrogen phosphate (0.1%), calcium carbonate (0.2%), magnesium sulphate heptahydrate (0.01%), ferrous sulphate heptahydrate (0.001%), zinc sulphate heptahydrate (0.001%), and copper sulphate pentahydrate (0.001%). The organism was grown in this medium at 28–30°C for 72–96 h.

### *Hydrolysis and extraction of fermentation broth*

A 5-ml volume of the whole broth was adjusted to pH 2 with 1 ml of 1 *N* hydrochloric acid and heated at 80°C for 60 min in a water-bath. The broth was then cooled to room temperature, mixed with 5 ml of chloroform–methanol (3:2) for 15 min on a shaker and centrifuged. The organic layer was removed, concentrated *in vacuo* and dissolved in 0.1 ml of ethanol. Depending on the expected concentration level, 2 or 4 μl were spotted on a TLC sheet.

### *TLC*

Silica gel 60 $F_{254}$ precoated TLC aluminium sheets (art. 5554, E. Merck, Darmstadt, F.R.G.) were impregnated by dipping them into a 0.5 *N* oxalic acid solution. The sheets were air-dried in a horizontal position overnight.

Samples and standard, dissolved in ethanol, were applied with a glass capillary (2 μl) in a 5-mm wide strip.

The chromatograms were developed with chloroform–acetone (7:3) in vapour-saturated 10 × 10 cm twin-through chambers (CAMAG, Switzerland), lined with filter paper. The running distance was 6 cm, thus the elution time was *ca.* 10 min at room temperature. Afterwards the sheets were air-dried. The separated red-

TABLE I

RECOVERY AND PRECISION

| *Fermentation broth level (μg/ml)* | *Recovery of adriamycin* (%)* |
|---|---|
| 2 | 81.5 ± 7.0 |
| 4 | 83.0 ± 5.7 |
| 10 | 80.0 ± 6.0 |
| 20 | 82.7 ± 6.0 |

* Mean ± S.D. ($n$ = 6).

dish-violet coloured adriamycinone zones were scraped off with a zone scraper (micro vacuum cleaner), eluted with 3 ml of ethanol and measured fluorimetrically.

*Evaluation*

Fluorimetric measurements were performed with a UV/VIS spectrophotometer Spekol with fluorescence equipment (Zeiss, Jena, G.D.R.) at 589 nm after excitation at 483 nm. Daunomycinone was used as standard because it has both a structural similarity to adriamycinone and an identical fluorescence spectrum. Calibration curves for standard solution in ethanol were linear over the concentration range studied, 0.2–2 μg/ml, and passed through the origin. The limit of sensitivity was *ca.* 0.2 μg/ml.

To confirm the practical utility of this semi-quantitative TLC analysis, we added known amounts of adriamycin to the fermentation broths of an anthracycline-nonproducing *Streptomyces* mutant and examined the recoveries (Table I).

Furthermore, in order to determine the precision and recovery of the TLC analysis itself without the extraction procedure of the fermentation broth, a standard solution (3 μg/ml) was directly chromatographed and estimated as described above. The mean recovery (± S.D.) was 88.97 ± 6.6% ($n$ = 7). Assuming that the recovery is normally distributed, we calculate the 95% confidence interval (82.85, 95.09) of its expectation.

## RESULTS AND DISCUSSION

TLC has been used to monitor anthracycline antibiotics in fermentation broths, but there are certain limiting factors, and often more than one mobile phase must be used for TLC because of the complexity of the fermentation mixture. Thus, during fermentation, adriamycin is formed together with many analogues, which include as main components daunomycin and its higher glycosides baumycins A and B, as well as 13-dihydrodaunomycin and ε-rhodomycinone. The whole broth was a complex mixture of at least eight to twelve components, but no adriamycinone could be detected in fermentation time. In particular, the separation of adriamycin from the stable 13-dihydrodaunomycin is difficult, because this anthracycline behaves identically with adriamycin in the most chromatographic systems. The hydrolysis step reduces the number of components to five, which can be separated completely as shown in Fig. 1. Only 7-deoxy-13-dihydrodaunomycinone, which is formed if the fermentation broth is kept at neutral pH in the absence of aeration for several hours,

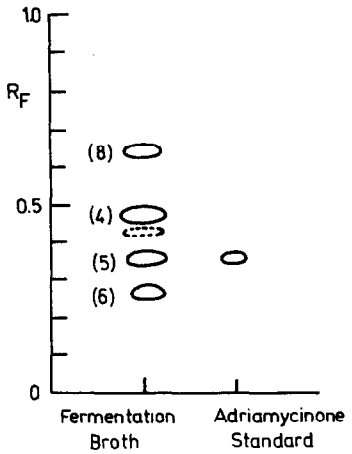



Fig. 1. Thin-layer chromatogram of extracted anthracyclinones from hydrolysed fermentation broth.

cannot be separated from adriamycinone in this system. As a control, an effective separation of these anthracyclinones is achieved in chloroform–methanol (95:5) on silica gel 60 $F_{254}$ precoated TLC aluminium sheets, impregnated with 0.5 *N* sodium hydrogen carbonate solution.

It can be concluded that the method described in this paper is sensitive and easy to use for the detection of adriamycin in anthracycline mixtures. It is simple, rapid, and economical, employing readily available materials and requiring little sophisticated equipment. Results are available within 2–3 h.

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