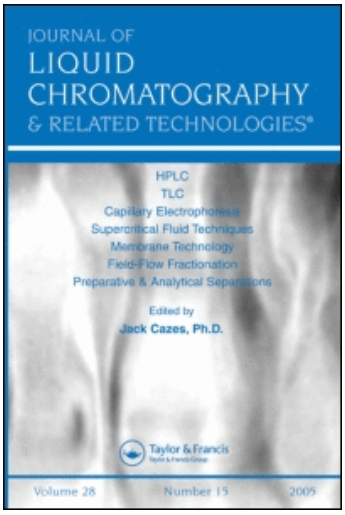


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# Quantitative Analysis of Tubercidin in *Streptomyces tubercidicus* Cultures by High Pressure Liquid Chromatography

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# QUANTITATIVE ANALYSIS OF TUBERCIDIN IN STREPTOMYCES TUBERCIDICUS CULTURES BY HIGH PRESSURE LIQUID CHROMATOGRAPHY

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## ABSTRACT

The tubercidin in Streptomyces tubercidicus cultures was extracted and detected by High Pressure Liquid Chromatography (HPLC). Using the methanol/water solvent (20/80), the column u-Bondapak C18 (Waters Associates) separated this antibiotic compound well. The detection was performed at 254nm where tubercidin was absorbed. This method provided a rapid and exact analysis for the amount of tubercidin present in cell free culture medium.

## INTRODUCTION

The tubercidin, 7-deaza-adenosine ribonucleotide, has antimycobacterial and antitumor activity (1). Tubercidin structure is analogous to that of adenosine and differs by virtue of its unique 7-deaza adnosine base as shown in figure 1.

There are several methods available for determining tubercidin. Smulson (2) described that mixtures of tubercidin, guanosine, etc. were separated by paper chromatography. And Dekker(3) and Uematsu(4) described that nucleotide antibiotics were separated by column chromatography.



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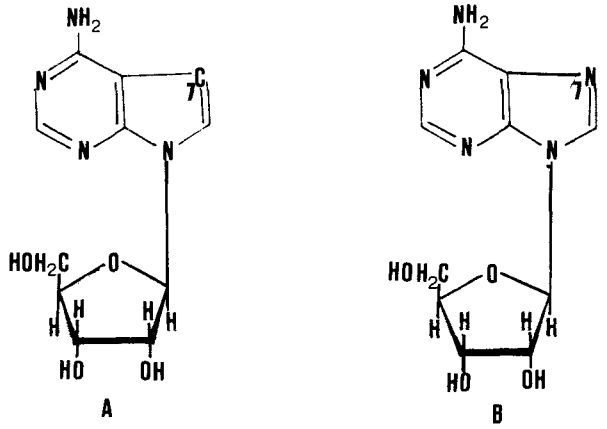


Figure 1

Structures of Tubercidin (A) and Adenosine (B)



The methods described above, however, were not suitable for the determination of tubercidin for several reasons; time-consuming, Complicated sample preparation and difficulty in exact quantitation in individual determination of biological mixtures. In this respect, a new method using HPLC for the quantitative analysis of tubercidin was developed in this present investigation.

## MATERIALS AND METHODS

### Bacterial Strains

S. tubercidicus ATCC 25502 which was obtained from Dr. Yim in our department, and three auxotrophic mutants, $phe^-$; $Ile^-$; $val^-$, $nico^-$; were used in this experiment. Three auxotrophic mutants were obtained from S. tubercidicus ATCC 25502 with UV and NTG by the procedure of Ochi et al. (5)

### Bacterial Culture Medium

a) Seed culture medium ; William's (6) peptone-yeast extract-glucose broth was used.

100ml of seed culture medium contained ; peptone, 0.5g; yeast extract, 0.2g; glucose, 1g; casein hydrolyzate, 0.1g; NaCl, 0.5g. 10ml of seed culture medium was poured into 100ml Erlenmeyer flasks. After the medium was autoclaved at 121'C for 15min and cooled down to room temperature, the test strains were inoculated.

b) Fermentation culture ; modified Vavra's (7) medium was used. Two litres of fermentation medium contained ; peptone, 10g; glycerol, 40g; ammonium sulfate, 5g; Calcium carbonate, 1g; 250ml of culture medium was poured into 1 litre culture flasks. S. tubercidicus seed media were maintained aerobically at 27'C on a rotatory shaker. After 2 days, 0.1% inoculum was added to 250ml fermentation medium.

Sample Preparation

Cell free filtrate sample preparation was performed on the basis of Smulson's method (2).

Sixty hours after inoculation, the medium was filtered by centrifugation (Hitachi Automatic Refrigerated Centrifuge, Hitachi Koki Co., LTD) to obtain cell free sample. Activated charcoal (1g/100ml) was added to the filtrate, after adjustment to pH 8.0 with $NH_4OH$ for 30min, and was removed by filtration. The charcoal was washed with 200ml of 80% acetone pH 2.0 (acidified with IN-HCl).

The aqueous-acetone solution was neutralized with ammonium hydroxide and taken dryness under vacuum. The residue was treated with hot absolute ethanol. The ethanol was evaporated to a small volume (5ml) and the insoluble material was removed. This sample solution was filtered through 0.45 um porosity nucleopore filter (Millipore corp.)

Standard Solution Preparation

Tubercidin (Sigma Co.) standard solution was prepared with 0.01mM and 0.1mM concentrations. Each guanosine, adenosine, cytosine (Sigma Co.) standard solutions were prepared with 0.1mM concentrations and standard mixture solution was prepared with several ratios of above standard solutions.

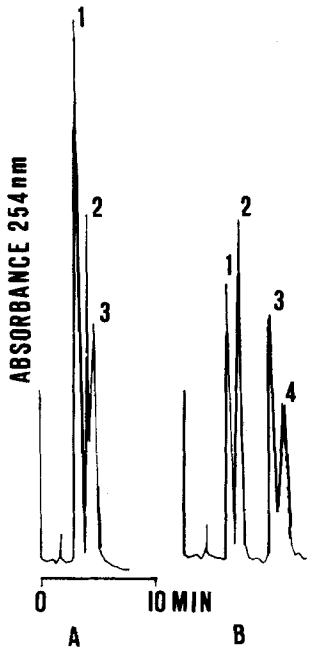


Figure 2

Tubercidin analysis pattern in standard mixture solution by HPLC using 30% Methanol solvent (A) and 20% Methanol solvent (B)
A 1, cytosine + guanosine ; 2, adenosine ; 3, tubercidine
B 1, cytosine ; 2, guanosine ; 3, adenosine ; 4, tubercidine



Analysis by HPLC

Operational conditions of HPLC (Waters Associates Inc. Milford, Mass 01757, USA) were as follow; column, u-Bondapak C18'; solvent, methanol/water (30/70, 20/80); flow rate, 1.0ml/min; detector U.V. model, 254nm; temperature, room temperature. Methanol solvents (for chromatographic grade, Merck) were degassed and filtered through Millipore filter prior to use.

## RESULT AND DISCUSSION

As tubercidin is analogous to necleoside, adenosine, we attempted to separate tubercidin from adenosine, tubercidin, cytosine,

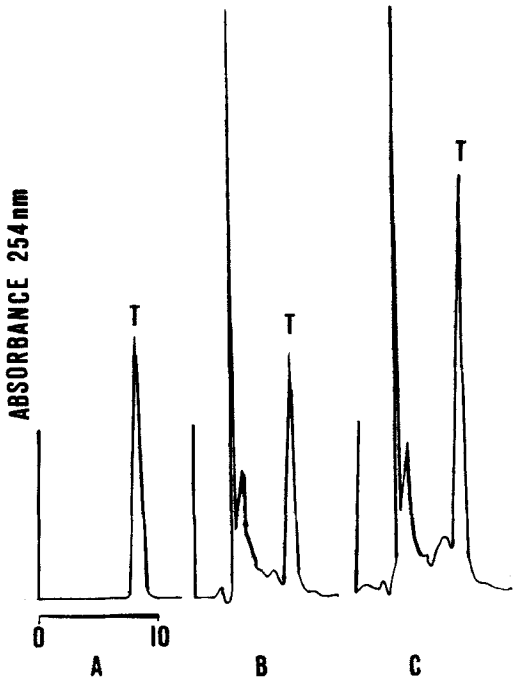


Figure 3

Tubercidin analysis pattern shown in cell filtrate sample by HPLC to show tubercidin peak (T) in tubercidin solution (A)[1], wild type cell filtrate solution (B)[2] and wild type cell filtrate solution plus tubercidin solution (C)[3].
1. 0.01mM tubercidin soln. 20 ul. 2. cell filtrate soln. 5 ul
3. cell filtrate soln. 5 ul + 0.01mM tubercidin soln. 20 ul

guanosine containing solution. Tubercidin in standard mixture solution could be well separated by HPLC using 20% methanol solvent could not separate four compounds completely as shown in figure 2-A. As shown in figure 3-B, tubercidin in cell filtrate samples was also detected by HPLC using 20% methanol solvent. To identify that the appeared peak is the true tubercidin one, standard tubercidin solution was added to the cell filtrate sample. The peak was increased as much as the amount of added tubercidin in turn as shown in figure 3-C. For exact quantitation of tubercidin, we

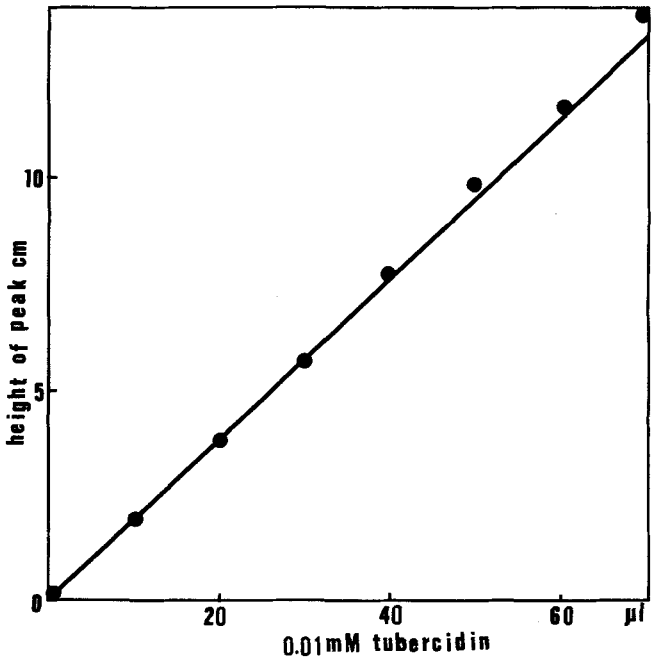


Figure 4

Tubercidin standard curve by HPLC chromatograms using 20% Methanol solvent. 10 ul aliguots of standard tubercidin solution (0.01mM) were injected.



prepared tubercidin standard curve by HPLC chromatograms using 20% methanol solvent, 0.01ml standard tubercidin solution. This result is shown in figure 4. On the basis of above result, we analyzed the tubercidin quantitatively in four strain culture filtrate samples. The result was as follow; Streptomyces tubercidicus ATCC 25502 (wild type), 0.51mg; $phe^-$ auxotroph, 0.28mg; $Ile^-$ auxotroph, 0.634mg; $val^-$, $Nico^-$ auxotroph 0.45mg per 100ml cell free filtrate samples. This result is shown in figure 5 to compare relative tubercidin amounts. One peculiar characteristic of above result was that $Ile^-$ auxotroph produced more tubercidin than wild type strain.

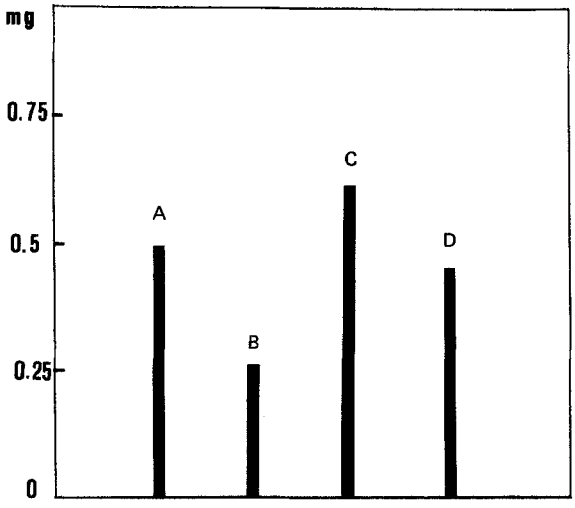


Figure 5

Relative tubercidin amounts produced by S. tubercidicus ATCC 25502 (wild type) and three auxotrophic mutants A, wild type strain; B, phe auxotrophic mutant ; C, Ile auxotrophic mutant ; D, val . nico auxotrophic mutant.
(mg/ 100ml cell filtrate samples)



This fact suggests that strain can be improved by mutation as published by Wesseling et al (8). Other peaks appeared in cell filtrate sample (Figure 3) would be removed by intense purification procedure using paper chromatography or column chromatography etc., but it seems that these peaks do not influence on the quantitative analysis of tubercidin. In summury, the HPLC method described above can be useful for the detection as well as quantitative analysis of tubercidin and other nucleoside antibiotics present in bacterial cultures.

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## REFERENCES

1. Buchanan, R. E., and N. E. Gibbon. Bergey's manual of determinative bacteriology. 8th ed. Williams & Wilkins comp. Baltimore 780 (1974)

2. Smulson, M. E., and R. J. Suhadolnik, J. Biol. Chem. 242(12), 2872 (1967)

3. Dekker, C. A., J. Am. Chem. Soc. 87, 4027 (1965)

4. Uematsu, F., and R. J. Suhadolnik, Biochemistry. 9(5), 1260 (1970)

5. Ochi, K., M. J. M. Hitchcock, and E. katz, J. Bacteriology, 139 (3), 984 (1979)

6. Williams, S. T., and T. Cross, Actinomycetes. In Method in Microbiology. C. Bootu, ed. 4 Academic Press, N. Y., 295 (1971)

7. Vavra, J. J., A. Dietz., and B. W. Churchill, Antibiotics and Chemotheraphy. 9, 426 (1959)

8. Wesseling, A. C., and D. L. Barbara, Developments in Industrial Microbiology. 20, 641 (1981)