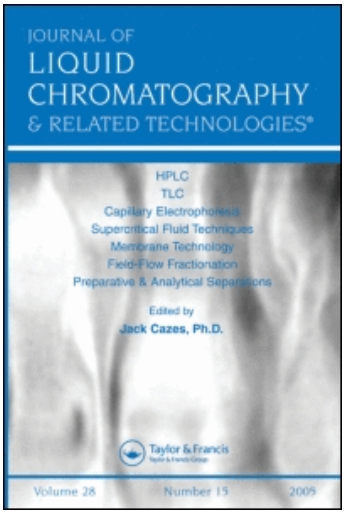

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HPLC DETERMINATION OF THE MAJOR FACTORS OF THE NOURSEOTHRICIN COMPLEX, AN ANTIBIOTIC FOR NONMEDICAL USES

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ABSTRACT

A liquid chromatographic method has been developed for the quantification of nourseothricin (NTC) in fermentation broths. The two major components D and F of the nourseothricin complex, were separated by HPLC using a C8 bond silica gel column and an aqueous ion pair solvent consisting of 0.02% octane-1-sulfonic acid sodium salt, 0.05% trifluoroacetic acid and 43% acetonitrile.

The individual components, as well as unknown minor constituents, were detected by UV 210 nm. It was decided to quantify NTC based on the sum of the peak areas of D and F. The analytical response was linear over a concentration range of 10 – 80 μg/mL.

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INTRODUCTION

Nourseothricin (NTC), a secondary metabolite complex produced by *Streptomyces noursei* ZIMET 43716, belongs to the group of streptothricin antibiotics. The complex consists of the main components F and D, as well as the minor components E and C. NTC is classified as a broad-spectral antibiotic because it inhibits the growth of both Gram-positive and -negative bacteria as well as mycobacteria and some types of fungi and viruses.[1,2] The mechanism of action involves the inhibition of ribosomal protein synthesis and induction of miscoding.[3] No cross resistance has been shown with antibiotics which are mainly used in human and veterinary therapy.

As the other streptothricins, NTC also contains three building blocks: the carbamylated amino sugar D-gulosamine, the lactam form of the heterocyclic amino acid streptolidine, and β-lysine. The individual components of NTC are distinguishable by the number of β-lysine groups connected with each other (Figure 1).

NTC was found to improve live weight gain and feed conversion in animal husbandry. Its low enteral absorption, intestinal degradation to inactive products, and early renal excretion are advantageous properties that are generally appreciated for an oral application.

Furthermore, NTC is a promising agent for agricultural uses. There are encouraging references in the field of plant protection, especially in prevention of phytopathogenic bacterial diseases. Those are, for instance, the applications against the fire blight (*Erwinia amylovora*) and in the cauterization of seed-potatoes against decaying during storing (*Erwinia carotovora ssp. atroseptica, Phytophthora infestans*).

Another nonmedical use of NTC is the following; together with the resistance genes *nat1*, *nat 2* (*Streptomyces noursei*), *sat1, sat2, sat3* (*Escherichia*

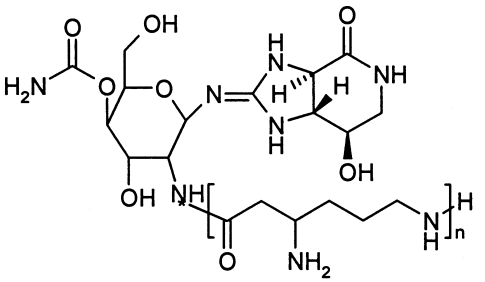

Figure 1. The structure of NTC C, D, E, and F.

coli), and *stat* (*Streptomyces lavendulae*), which encode nourseothricin-N-acetyltransferases, NTC can be used as a selection system in molecular genetics of microbes and plants.[4,5] The above genes, and their products, confer resistance to the host by monoacetylation of the β-amino group of the ß-lysyl moiety. The resistance genes are available as markers for heterologous expression. NTC may be used in fermentations of recombinant clones to maintain the permanent propagation of vectors carrying at least one of the resistance genes.

Traditionally, a microbiological method has been employed for the detection and quantification of NTC in biological matrices,[6] however, this method lacked speed and specificity. Microbiological techniques have little or no potential for detection and quantification of metabolites of the parent drug. Numerous chemical and physical methods have been reported for the determination of NTC.[7,8] None of these methods is specific for NTC. Liquid chromatography is increasingly used as a method for the determination of antibiotics in biological matrices.

This paper reports the development of a HPLC method for the detection of NTC in fermentation broths and water solutions. Since there is no significant difference in the structure of the NTC components, the separation of the components is difficult. However, a sufficient separation can be achieved using ion-pair chromatographic technique. This method was extended to the examination of the fermentation broth as well, assuming the fast and reliable control of antibiotic level. A typical chromatogram of NTC is shown in Figure 2. It was decided to quantify NTC based on the sum of the peak areas of the NTC components D and F.

EXPERIMENTAL

Reagents

All chemicals were obtained from commercial sources. Solvents were HPLC-grade. Acetonitrile was purchased from Riedel-de Haën (Germany). Demineralized water (Milli-Q, Millipore, Germany) was used. Octane-1-sulfonic acid Na-salt (Lichropur®) and trifluoro-acetic acid (Uvasol®) were obtained from Merck (Germany). The standard NTC sulfate was produced in the Hans-Knöll-Institute for Natural Product Research.

Liquid Chromatographic Determination

The analyses were performed with a Shimadzu HPLC system consisting of two LC-7A pumps, a CBM-10A communications bus module, a SPD-M10A photodiode array detector, a CTO-10A oven with gradient mixer, a LC workstation with CLASS LC10 software, a manual Rheodyne 7125 sample injector with 20 μL loop.

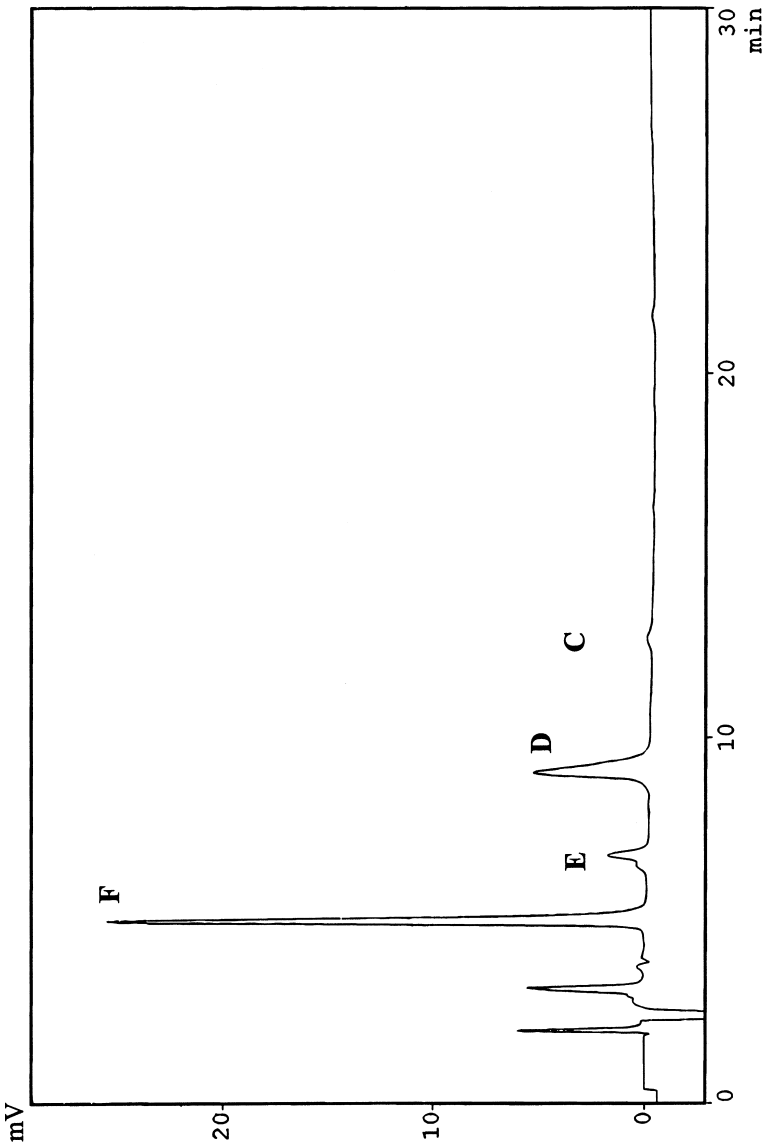

Figure 2. Chromatogram of a standard solution of NTC.

The composition of the eluent was 0.05% TFA, 0.02% octane-1-sulfonic acid Na-salt, 43% acetonitrile in water, filter mobile phase, and deaerate by vacuum draw-off, flow rate 1 mL/min. The separation was accomplished on a Lichrospher 100 RP-8, 5 μm column, 250 × 4.6 mm ID. (Merck, Germany).

The detection was carried out at 210 nm. The two major components F and D have retention times of 4.8 and 9.1 minutes. The quantitative determination was achieved according to a calibration curve by measuring of the peak area.

The analysis of the NTC components D and F in the fermentation broth allowed the monitoring of the antibiotic level upon fermentation.

Preparation of the Sample

Taking samples every 24th hour, it was established that no detectable NTC substance was present after 24 hours of the inoculation of the producing strain *Streptomyces noursei* ZIMET 43716. After that time, the antibiotic level quickly increased and reached the maximum after 110 – 120 hours. At this point, the fermentation was stopped and and the broth was worked up.

5 mL of the fermentation broth were centrifuged 10 min at 3600 × g and an aliquote of the supernatant was further diluted with water (1:100) to give working solutions of 10 – 20 μg/mL. 20 μL aliquots were injected onto the HPLC column.

NTC Standard Solution

A 1.4245 mg NTC-sulfate standard were dissolved in 1 mL water. This solution contains 1 mg NTC-base per mL and was used as the stock solution. For HPLC calibration, the stock solution was diluted with a fermentation broth at 0 hour to obtain standard working solutions of 10, 20, 40, and 80 μg/mL, respectively.

RESULTS AND DISCUSSION

Method Validation

The analytical peaks of NTC F and D were well resolved (Figure 2), and the mean ± S.D. (n=6) of retention times were 4.8 ± 0.028 for F and 9.1 ± 0.053 for D. No endogenous peaks were observed which might interfere with the peak areas measurement of the analytical peaks in the fermentation broths. The run time was set at 30 min in order to eluate endogenous components before the next run.

Table 1. Precision and Recovery for the Determination of NTC*

Actual Concentration (μg/mL)	Mean Measured Concentration (μg/mL) and Recovery (%) (n=6)	Relative Standard Deviation
15	14.6 (97.3%)	6.2%
30	34.4 (114.6%)	4.3%
40	36.8 (91.6%)	5.9%
50	55.8 (111.6%)	4.6%
75	78.0 (104.0%)	5.0%

*The calibration curve was studied within one day.

The data obtained from the quality-control samples assayed for validation of the method are shown in Table 1. Calibration curves for the NTC components D and F were obtained by plotting the sum of the peak areas of D and F versus the antibiotic concentration of the NTC complex. Two calibration curves spanning lower (10 – 40 μg/mL) and higher (40 – 80 μg/mL) concentration ranges were prepared to cover a wide range of detection from unknown samples. Each concentration of NTC was analyzed six times. Figure 2 shows a typical chromatogram of a NTC standard solution using the condition described above. The calibration curves routinely yielded correlation coefficients of >0.99.

The linearity was studied in the range of 10–80 μg/mL NTC, corresponding to the ranges observed in fermentation samples. The relationships between peak areas (NTC F + D) were linear over this range.

The data presented in Table 1 showed recovery from 91 to 114%. The precision was assessed from peak areas measurements at five concentrations. The limits of detection (LOD) and quantification (LOQ) of the assay were determined by measuring the noise of a control sample. The LOD was 0.1 μg/mL. For this minimum detectable concentration, a signal-to-noise ratio of approximately 3:1 was observed. The LQD was evaluated as the concentration equal to 10-times the value of signal-to-noise ratio. It was evaluated as 2 μg/mL for practical measurements.

The accuracy (expressed by the deviation between found and added concentration) was assessed at different concentrations. Actual concentrations were compared to expected concentrations, and the relative error was calculated. The results of accuracy, presented in Table 1, ranged from −5.9 to 5%.

In conclusion, the assay procedure described in this report can successfully be applied for the determination of the major components D and F of NTC in fermentation broths. The HPLC method presented here is rapid, sensitive, specific, and robust. This assay might be employed in the analysis of NTC concentrations in other biological samples.

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