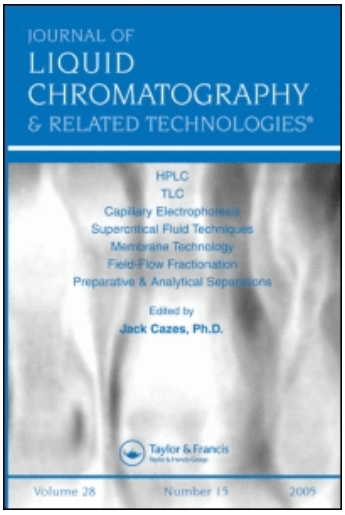

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High-Performance Liquid Chromatography of Derivatives Involved in the Terminal Steps of Tetracycline Biosynthesis in *Streptomyces Aureofaciens*

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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF DERIVATIVES INVOLVED IN THE TERMINAL STEPS OF TETRACYCLINE BIOSYNTHESIS IN STREPTOMYCES AUREOFACIENS

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ABSTRACT

A simple and reproducible method was developed for the analysis of tetracycline derivatives involved in the last two steps of tetracycline biosynthetic pathway in *Streptomyces aureofaciens*. The method is based on a gradient liquid chromatographic separation of the compounds using a microbore octadecyl silica column. Beside separation of a mixture of standards, the method was used for separation, detection and quantitation of dehydrotetracycline and tetracycline prepared enzymatically *in vitro* using anhydrotetracycline oxygenase and tetracycline dehydrogenase, respectively, isolated from *S. aureofaciens*. The method permits a simple and accurate characterization of kinetics of corresponding enzymatic activities.

INTRODUCTION

Tetracycline biosynthesis in *Streptomyces aureofaciens* proceeds in a sequence of reactions catalyzed by specific enzymes from which those involved in the terminal steps have been detected and partially characterized. These include S-adenosylmethionine:dedimethylamino-4-aminoanhydrotetracycline N-methyltransferase that catalyzes methylation of dedimethylamino-4-aminoanhydrotetracycline yielding anhydrotetracycline (ATC; 1), anhydrotetracycline oxygenase (ATCox) converting ATC to dehydrotetracycline (DHTC; 2-5), and NADP:tetracycline 5a(11a)-dehydrogenase (TCdh) catalyzing the formation of tetracycline (TC; 6,7), the two latter ones being shown in this scheme:

It is not always easy to detect the ATCox and TCdh reactions directly by conventional spectrophotometric methods as DHTC is relatively unstable and the UV/Vis absorption bands of ATC, DHTC and TC overlap. To avoid this drawback, we developed a method of high-performance liquid chromatographic (HPLC) separation, detection and quantitation of the derivatives in question, i.e. ATC, TC and DHTC, and applied it to a real enzymatic system of *S. aureofaciens*. Moreover, to record UV/Vis spectra permitting a real-time detection of individual peaks and so avoiding the necessity of frequent column recalibration, we included a diode-array detector into our HPLC set-up.

MATERIAL AND METHODS

Chemicals.

TC was from the Institute of Antibiotics and Biotransformations (Roztoky near Prague, Czechoslovakia). ATC was prepared from TC by a procedure of Schlecht and Frank (9). DHTC was prepared from ATC enzymatically using partially purified ATCox from *S. aureofaciens* as described further. The standards were dissolved in a minimum volume of MeOH, diluted with the mobile phase and passed through a 0.45-μm Millex filter unit (Millipore). Glucose 6-phosphate, glucose 6-phosphate dehydrogenase and NADP were from Serva, Sephadex® G-25 and Phenyl-Sepharose® CL 4B were from Pharmacia LKB.

High-Performance Liquid Chromatography

An advanced Hewlett-Packard 1090M HPLC system was employed consisting of a binary solvent delivery system, an autosampler equipped with a 25-μl autoinjector, a diode-array detector operating between 190 and 600 nm and the workstation comprising a Hewlett-Packard 310 technical computer, an HP 9133 710 kB/20 MB dual disc drive, an HP 7475 plotter and an HP 2225A ThinkJet printer. The HPLC data were acquired and the raw data processed using a standard Hewlett-Packard HPLC software package, revision 3.0.

Run were performed at 40 °C on a Separon octadecyl silica glass-pack column, 150 x 1 mm i.d., 5 μm particles (TESSEK, Prague, Czechoslovakia), with gradient elution. The mobile phase "A" contained dimethyl formamide/water (20 mM EDTA), 20:80, pH 6.4, the "B" phase was 100% MeOH. Flow rate of 0.1 ml/min was changed to 0.2 ml/min at time 1 min and after 2 min reduced to the initial value. A 0.5-min linear gradient of 0 to 50% "B" was started at time 0.5 min. The 50% concentration of "B" was held for 2 min, then a 0.5-min gradient to 0% "B" was performed. A 1.5-min equilibration delay was allowed before the next sample injection.

Cultivation of *S. aureofaciens* and Sample Preparation

Streptomyces aureofaciens strain 50/137 derived from the strain 84/25 (Research Institute of Antibiotics and Biotransformations, Roztoky near Prague, Czechoslovakia)

was cultivated and the cell-free extracts prepared as described elsewhere (2,4).

The enzymatic preparation of DHTC and TC was carried out employing whole *S. aureofaciens* cell-free extract passed through a Sephadex G-25 column or a partially purified ATCox or TCdh preparation. Partial purification of ATCox and TCdh resulting in their separation from each other was achieved by hydrophobic interaction chromatography of cell-free extract on a Phenyl-Sepharose CL-4B column (4,5).

The reaction mixtures contained in a total volume of 0.5 ml 0.24 mM NADP, 0.6 mM glucose 6-phosphate, 1.1 U glucose 6-phosphate dehydrogenase, 0.08 M Tris-HCl (pH 7.4), 160 μM ATC and an appropriate enzymatic preparation. In the case of the ATC-to-TC conversion, either whole cell-free extract or the two partially purified enzymes in combination were added to the mixture. DHTC was the end product when partially purified ATCox free of TCdh was employed in the reaction. Finally, partially purified TCdh was in the DHTC-to-TC conversion reaction mixture. The mixtures containing partially purified ATCox were also supplemented with 50 μl deproteinized *S. aureofaciens* cell-free extract (4).

The reactions were performed at 29-30 °C and allowed to reach a complete substrate-to-product conversion. Aliquots were withdrawn from the reaction mixture after completion of the corresponding reaction, passed through a 0.45-μm Millex filter unit and directly injected onto the HPLC column. In the case of analysis of the mixture containing whole cell-free extract (ATC-to-TC conversion system), an aliquot was also analyzed that was taken from the mixture in the stage of maximum content of DHTC which is an intermediate in this enzymatic system. The time delay between sample withdrawal in this case and its injection was negligible compared to the reaction rate.

RESULTS AND DISCUSSION

In FIGURE 1A, an HPLC separation is shown of a mixture of standards, where 1 stands for DHTC, 2 for TC and

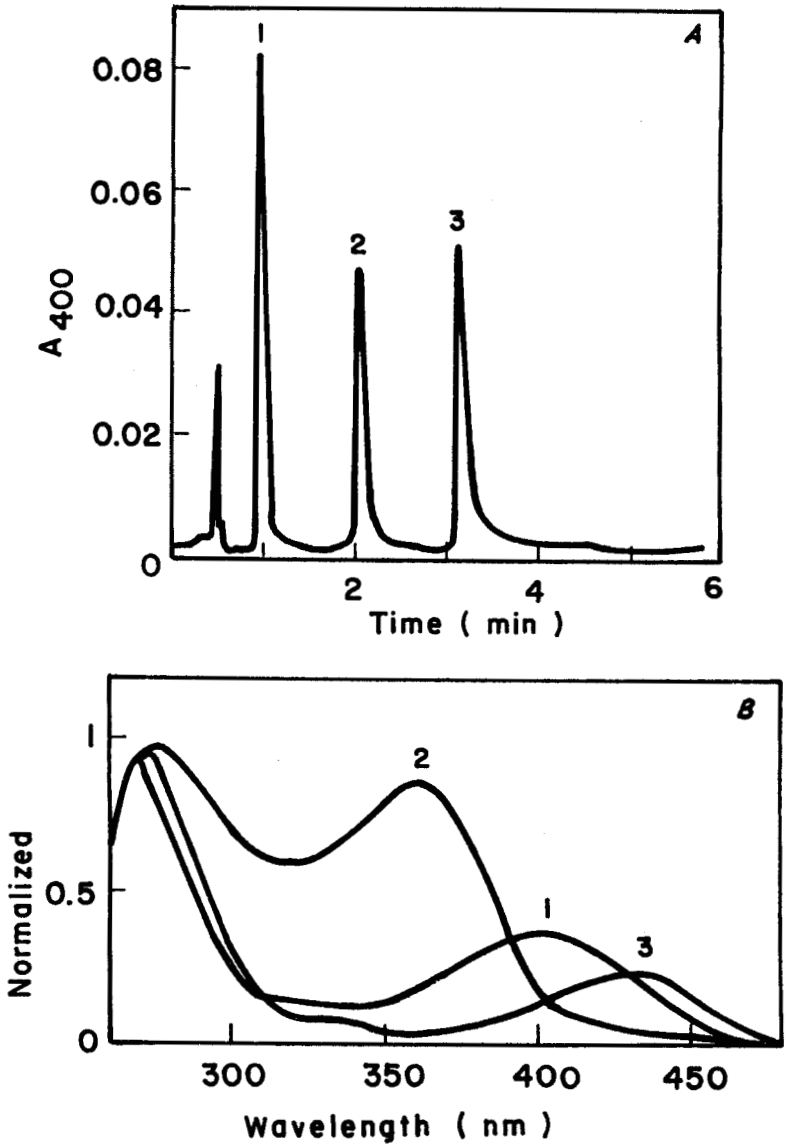

FIGURE 1 **A** - Reversed-phase HPLC of a mixture of standards (5 µl), monitored at 400 nm. The numbers 1, 2 and 3 stand for DHTC, TC and ATC, respectively.

B - Real-time spectra of peaks 1, 2 and 3.

HPLC conditions are given under MATERIAL AND METHODS.

3 for ATC. The peaks are well resolved under our conditions. The flow rate in the first minute was lower (0.1 ml/min) than later (0.2 ml/min) to resolve the DHTC peak from impurities eluted in the front. The higher flow rate starting at 1 min as well as the MeOH gradient considerably reduced the retention time of both TC (2.1 min) and ATC (3.2 min). Under initial conditions, i.e. flow rate of 0.1 ml/min and isocratic elution at 100% "A", kept constant during the whole run, the retention time was about 8 min and 16 min for TC and ATC, respectively (not shown). Moreover, under these conditions, considerable tailing of the ATC peak occurred necessitating the insertion of at least 5-min equilibration delay following the ATC peak apex that would result in more than 20-min runs. The higher flow rate and MeOH gradient led not only to a more than 5-fold ATC retention time reduction but also improved the peak tailing favouring easier and more accurate quantification as with fairly tailing peaks quantification based on peak area is rather error-prone. Hence the employed HPLC conditions allow both short-time runs and accurate quantification and are suitable for multiple-injection analyses.

Real-time absorption spectra corresponding to the resolved peaks in FIGURE 1A are displayed in FIGURE 1B. Recording of the spectra was facilitated by the employment of a diode-array detector with a 20-ms sampling interval. The absorbance maxima in the visible range, i.e. 360, 400 and 432 nm for TC, DHTC and ATC, respectively, fairly correspond to the published values for these compounds (9,10). The DHTC spectrum (FIGURE 1B, 1) is in accord with its sketchy description given elsewhere (10)

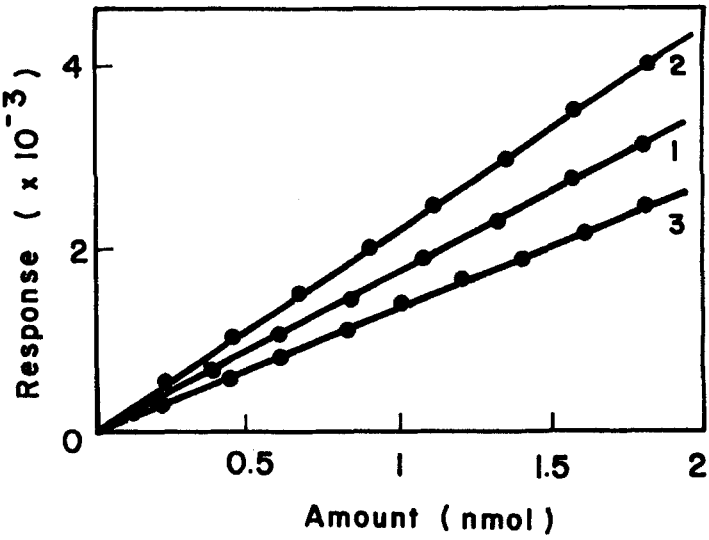

FIGURE 2 Calibration curves of DHTC (1, 400 nm), TC (2, 360 nm) and ATC (3, 440 nm). Details see under RESULTS AND DISCUSSION.

representing the only data on UV/Vis spectrum on DHTC published so far.

The mobile phase we used was adapted from another source (11). However, we omitted oxalic acid in the mobile phase "A" as this additive, due to its precipitation in the presence of organic solvents, precluded the use of MeOH gradient. The omission of oxalic acid had no significant effect on the retention time values or peak broadening.

The calibration curves (FIGURE 2) were constructed as plots of peak areas versus the amounts injected. The quantifications were, thanks to the diode-array detector, accomplished from chromatograms recorded at the wave-

lenght corresponding to the absorbance maximum wavelength for TC and DHTC, i.e. 360 and 400 nm, respectively. This feature increased the selectivity of the method and enhanced the signal-to-noise ratio that is of crucial imimportance at very low doses. ATC was quantified at 440 nm which is the wavelength of the ATCox spectrophotometric assay (2). Linearity of response was achieved from 0.1 nmol through 5 nmol of each compound.

The application of the above method to a real reaction mixture is shown in FIGURE 3. In all cases, 5-μl aliquots were analysed. FIGURE 3A presents an HPLC analysis of a sample from the reaction mixture containing whole desalted cell-free extract of *S. aureofaciens* mycelium withdrawn in the stage of maximum DHTC content. FIGURE 3B displays a chromatogram of an aliquot taken from the same reaction mixture as above after a complete ATC-to-TC conversion. The analysis of the reaction mixture containing partially purified ATCox free of TCdh is displayed in FIGURE 3C. FIGURE 3D shows an analysis of a sample from a mixture containing partially purified TCdh. In this case, the substrate of the reaction was DHTC. Real-time spectra of peaks 1 and 2 from FIGURE 3A-D are seen in FIGURE 3E. As expected, peak 1 corresponds to DHTC and peak 2 to TC.

Comparison of data displayed in FIGURE 3A-E with those in FIGURE 1A,B indicates that the HPLC method presented in this communication is applicable for separation, detection and quantification of the substrates and products in a real reaction mixture involving the ATC-to-DHTC, DHTC-to-TC or complete ATC-to-TC conversion. The method is rapid (allowing as much as 12 runs in an hour),

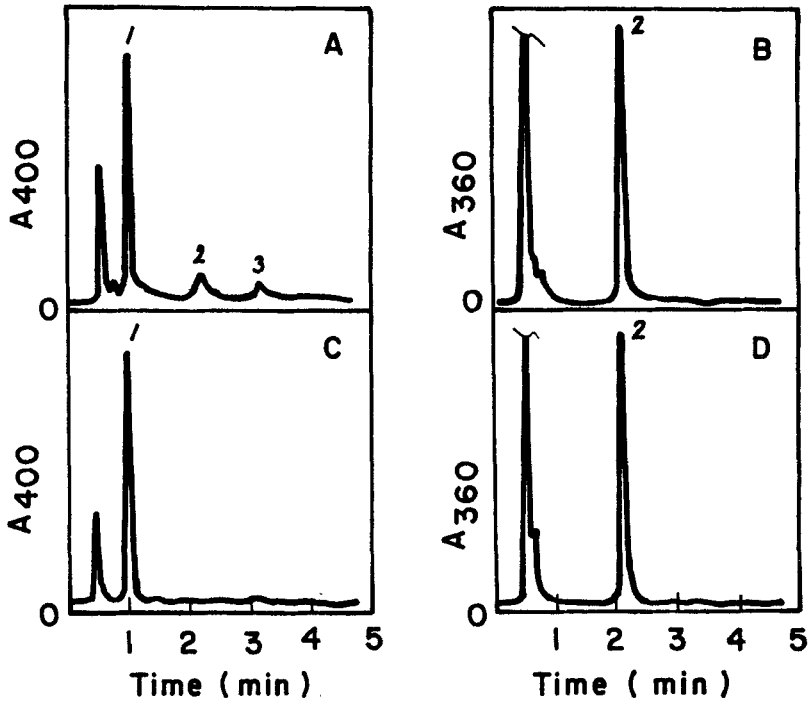

FIGURE 3 Reversed-phase HPLC analysis of real reaction mixture.

A - Analysis of reaction mixture with whole cell-free extract at the stage of maximum DHTC content.

B - Analysis of reaction mixture with whole cell-free extract after termination of the ATC-to-TC reaction.

C - Analysis of reaction mixture containing partially purified ATCox after termination of the ATC-to-DHTC reaction.

D - Analysis of reaction mixture containing partially purified TCdh after termination of the DHTC-to-TC reaction.

E - Real-time spectra of peaks 1 and 2.

HPLC conditions are given under MATERIAL AND METHODS.

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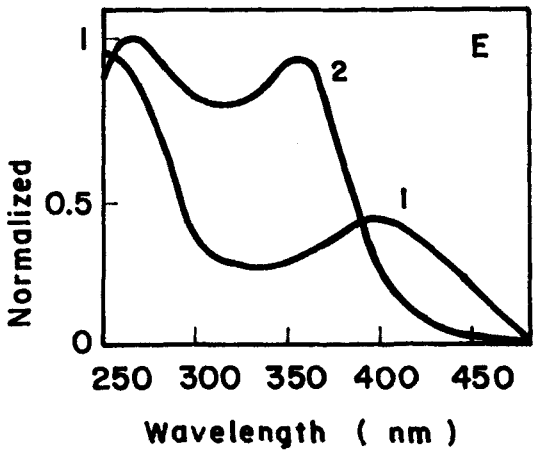

FIGURE 3 (continued)

reproducible and is suitable for automated multiple-injection sequences including raw data processing.

Another favourable feature of this method is that it allows direct analysis without extraction of the sample. As follows from FIGURE 3A-D, all the "impurities" are eluted in the front peak so they do not impair quantification of DHTC. These impurities, containing a lot of biological macromolecules, lead to column clogging and backpressure rise (approximately 100 injections per column reasonable), so the usage of a low-priced glass-pack column is advantageous.

It follows from the above that this method is a good starting point for establishment of an HPLC method of direct-injection monitoring of the ATC-to-DHTC, DHTC-to-TC and ATC-to-TC reactions which is the subject of our current studies.

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