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Note

High-performance liquid chromatographic procedures in monitoring the production and quality control of chlortetracycline

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Some tetracycline compounds, especially chlortetracycline, appear as more or less tailing peaks in different reversed-phase high-performance liquid chromatographic (HPLC) methods[1–3]. Reversed-phase column performance depends on the properties of the silica gel used and on the bonding technique. Because different reversed-phase packings are available, it is essential to select them carefully; a phase that is perfect for one separation may be inadequate for another. Performance also varies with the chromatographic conditions and the solutes under examination[4].

In this work, we tried to select the most suitable and simplest HPLC method for the precise and accurate determination of chlortetracycline and related tetracyclines that arise during biosynthesis in chlortetracycline production.

EXPERIMENTAL

Chromatographic system and methods

The isocratic mode was used for all experiments. The HPLC system consisted of an LKB 2150 pump, LKB 2151 variable-wavelength monitor, LKB 2220 computing integrator and a Rheodyne Model 7125 loop injector with a 20-μl fixed loop. Integration was based on peak-area measurement and determinations were carried out by the external standard method. Separations were made at ambient temperature. Degradation products of tetracycline and chlortetracycline were identified according to procedure of McCormick[5]. The columns used were LiChrosorb RP-8, 5-μm irregular particles (250 × 4.6 mm I.D.), TSK ODS 120 A, 5-μm particles (250 × 4.6 mm I.D.) (Merck, Darmstadt, F.R.G.), and Waters Nova-Pak C_{18} (75 × 3.9 mm I.D.) (Waters Assoc., Milford, MA, U.S.A.).

For different types of separations the following mobile phases and conditions were applied: 0.2 *M* citric acid + tetrabutylammonium hydroxide (0.5 g/l) (pH 2.5)–acetonitrile (92:8), UV detection at 360 nm; 0.05 *M* diethanolamine + 0.001 *M* disodium EDTA (pH 7.3)–isopropanol (88:12), UV detection at 360 nm; and methanol–acetonitrile–0.01 *M* oxalic acid (1:1.5:4), UV detection at 365 nm.

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Chemicals

Acetonitrile, methanol and tetrabutylammonium hydroxide (TBA) (20% aqueous solution) were obtained from Merck and oxalic acid dihydrate and sodium

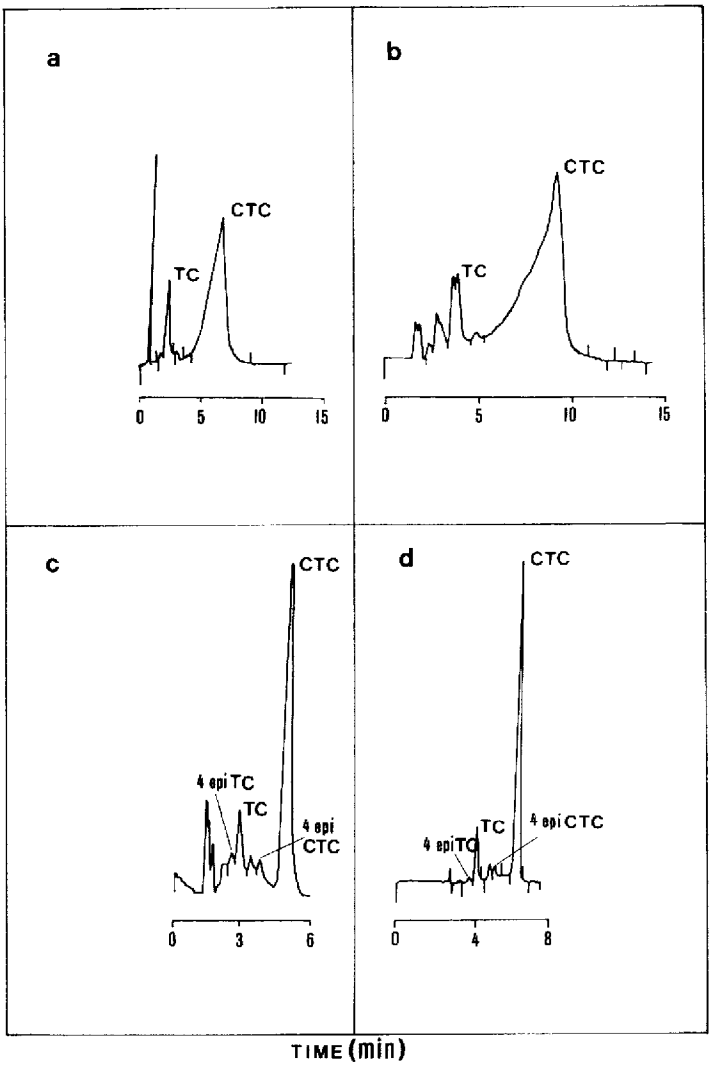

Fig. 1. Influence of different HPLC procedures on the separation of chlortetracycline referene standard. (a) Column, LiChrosorb RP-8, 5 μm (250 × 4.6 mm I.D.); mobile phase, 0.2 *M* citric acid + TBA (0.5 g/l) (pH 2.5)–acetonitrile (92:8); flow-rate, 1 ml/min; detection, UV at 360 nm. (b) Column, Waters Nova-Pak C_{18} (75 × 3.9 mm I.D.); mobile phase, 0.05 *M* diethanolamine + 0.001 *M* disodium EDTA (pH 7.3)–isopropanol (88:12); flow-rate, 0.5 ml/min; detection, UV at 360 nm. (c) Column, Waters Nova-Pak C_{18} (75 × 3.9 mm I.D.); mobile phase, methanol–acetonitrile–0.01 *M* oxalic acid (1:1.5:4); flow-rate, 0.5 ml/min; detection, UV at 365 nm. (d) Column, TSK ODS 120 A, 5 μm (250 × 4.6 mm I.D.); mobile phase, methanol–acetonitrile–0.01 *M* oxalic acid (1:1.5:4); flow-rate, 1 ml/min; detection, UV at 365 nm. Peaks: TC = tetracycline; 4-epi TC = 4-epitetracycline; CTC = chlortetracycline; 4-epi CTC = 4-epichlortetracycline.

ethylenediaminetetraacetate (EDTA) (both of analytical-reagent grade) from Kemika (Zagreb, Yugoslavia). Water for mobile phase preparation was deionized and distilled. Pure standards of chlortetracycline and tetracycline was obtained from the Institution for Drug Control and Testing (Zagreb, Yugoslavia). Different type of chlortetracycline samples were supplied by Krka Pharmaceuticals (Novo Mesto, Yugoslavia).

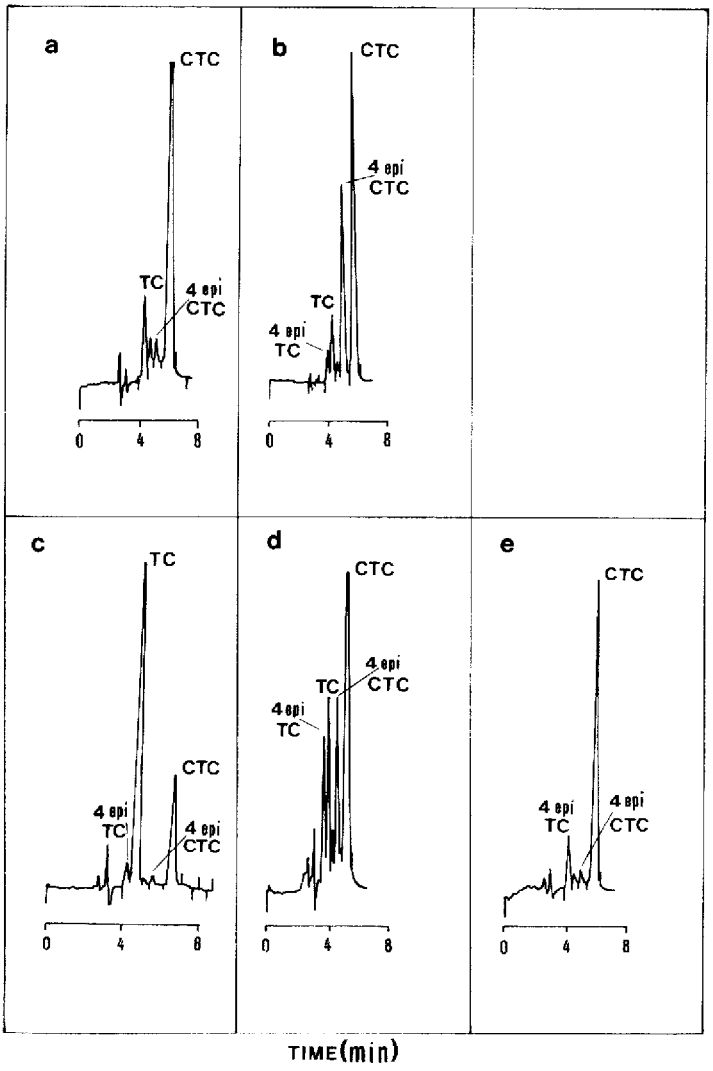

Fig. 2. HPLC separation of chlortetracycline by the optimum procedure. Column, LiChrosorb RP-8, 5 μm (250 × 4.6 mm I.D.); mobile phase, methanol–acetonitrile–0.01 *M* oxalic acid (1:1.5:4); flow-rate, 1 ml/min; detection, UV at 365 nm. Samples: (a) chlortetracycline reference standard; (b) chlortetracycline reference standard exposed to degradation procedure according to McCormick[5]; (c) chlortetracycline fermentation broth (low level of chlortetracycline); (d) chlortetracycline product (high level of 4-epichlortetracycline); (e) chlortetracycline product (proper quality, with level of 4-epichlortetracycline not higher than 5%). Abbreviations as in Fig. 1.

RESULTS AND DISCUSSION

In biotechnological processes for chlortetracycline production, a series of different tetracycline substances usually arise[6]. The fraction of tetracycline is produced simultaneously with chlortetracycline. The amounts of the degradation products of these two compounds such as 4-epitetracycline and 4-epichlortetracycline vary in relation to selected technological parameters. By using a suitable HPLC procedure, the appearance and monitoring of these compounds can be precisely studied during biotechnological processes.

For this purpose some different reversed-phase HPLC methods and columns were tested. It was found that the composition of the mobile phase played an important role in solving this problem[7]. In testing different types of reversed-phase column, it was found that columns of the highest quality and performances were not necessary if an appropriate mobile phase was chosen. Addition of TBA[3] and EDTA[1] to a particular mobile phase did not show the expected effects on tailing peaks (Fig. 1a and b). However, the addition of oxalic acid in an appropriate concentration acts as a very good complexation agent that prevents bonding of tetracycline substances to the reversed-phase material, thus preventing tailing effects[7] (Fig. 1c and d and Fig. 2).

The best results were obtained on a TSK ODS 120 A (5 μm) column (250 × 4.6 mm I.D.) and elution with methanol–acetonitrile–0.01 *M* oxalic acid (1:1.5:4) (Fig. 1d). The fully end-capped Waters Nova-Pak C_{18} column (75 × 3.9 mm I.D.) did not give a significant improvement in peak shape and resolution using the same mobile phase (Fig. 1c). The LiChrosorb RP-8 (5 μm) column with irregular particles (250 × 4.6 mm I.D.) gave satisfactory results, and this column with methanol–acetonitrile–0.01 *M* oxalic acid (1:1.5:4) as the mobile phase was selected for the simple and accurate monitoring of chlortetracycline production during the fermentation process and to establish quality control parameters for the final products (Fig. 2). With this method it proved possible to separate the tetracycline compounds and some of their degradation products in a relatively short time and with good symmetry of all peaks.

The appearance of the tetracycline fraction in the biosynthetic process is strongly reduced by the addition of a suitable concentration of Cu^{2+} together with Cl^- ions in the fermentation media. Simultaneously with reduction of tetracycline production, appropriate concentration of Cu^{2+} and Cl^-, induce high levels of chlortetracycline production[8] (Fig. 2b and c).

It is also possible to control the quality of chlortetracycline products with respect to the appearance of 4-epichlortetracycline as a degradation product and microbiologically inactive form of chlortetracycline. The epimerization of chlortetracycline to 4-epichlortetracycline[9] could be clearly demonstrated during the recovery procedure, whereas this process is not significant in the fermentation phase. Temperature seems to play an important role in the epimerization reaction, as this reaction is diminished when cooling is applied (Fig. 2d and e).

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