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Separation and quantitative determination of tetracycline, epitetracycline, epianhydrotetracycline and anhydrotetracycline by high-performance liquid chromatography

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SUMMARY

A high-performance liquid chromatography (HPLC) separation of tetracycline and its degradation products on a C_{18}-reversed phase column with the mobile phase water–acetonitrile–perchloric acid in two steps with varying amounts of acetonitrile, is described. Six different tetracycline preparations analyzed contain these impurities from undetectable to 7.78%. The detection limit was found to be about 5 μg/ml and the relative standard deviation of the method is between ±0.8% and 3.7%.

The broad-spectrum antibiotic tetracycline (TC) contains as impurities a number of related compounds, such as epitetracycline (ETC), epianhydrotetracycline (EATC) and anhydrotetracycline (ATC). Besides, these impurities possess almost no or very slight microbiological activity. Although the toxicity of EATC is established the exact safety level in preparations for human use is not known. An upper limit for these degradation products has been fixed by European pharmacopea.

High-performance liquid chromatography (HPLC) has already been applied by a number of authors to separate ETC, EATC and ATC from tetracycline bulk, where cation-exchange column with EDTA-buffer and C_{18} column with phosphate buffer and a linear gradient of 10–60% acetonitrile were used (Lindauer et al., 1976; Tsuji and Robertson, 1976). Knox and Jurand (1975) tried a number of columns and found an ODS (octadecylsilane), which had been previously treated to substitute any residual Si-OH groups with short chain trialkylsilyl groups (ODS/TAS), and a thoroughly silanized silica gel (Partisil) column (SC/TAS) very useful for separation of tetracyclines.

In the present work a reversed phase column packed with silica gel SI-100 (Merck) coated with a chemically bonded C_{18} organic phase was used for the separation of tetracyclines. An eluent system, water–acetonitrile–perchloric acid, has been used in two steps, with varying amount of acetonitrile, to separate ETC from TC and EATC + ATC

from TC bulk in 12 and 10 min, respectively. The method was tested for the analysis of six different tetracycline formulations. The separation of impurities and their quantitative determination was possible down to the range of about 0.5%.

Apparatus and reagents. A Perkin Elmer liquid chromatograph 1220 with a fixed wavelength (254 nm) detector and 1 mV Varian Aerograph recorder Model 20 was used. A Hewlett-Packard laboratory data system 3352 C was connected to the liquid chromatograph through an A/D convertor; reversed-phase column, 0.25 m × 2.6 mm i.d., packed with silica gel SI-100 (Merck), 10 μm, coated with octadecylsilane chemically bonded organic phase. All chemicals used were of reagent grade; acetonitrile was redistilled.

The mobile phase I for separation and determination of TC and ETC was: water–acetonitrile–perchloric acid (70%) 76.2 : 22 : 1.8. Mobile phase II for determination of EATC and ATC was: water–acetonitrile–perchloric acid (70%) 61.2 : 37 : 1.8. Tetracycline hydrochloride, epitetracycline hydrochloride, epianhydrotetracycline hydrochloride and anhydrotetracycline hydrochloride were obtained from European Pharmacopea Commission, Strasbourg, and Paul-Ehrlich-Institut, Frankfurt, in the best available quality. Six different pharmaceutical preparations containing tetracycline were analyzed.

Standard solutions. Fresh solutions of all four compounds in redistilled water were prepared every day. About 5 mg of each substance were accurately weighed, dissolved in redistilled water and were further diluted to give concentrations of 20, 50 and 100 μg/ml. A total of 5 μl were injected, four times each, into the liquid chromatograph and the integrated peak areas obtained from the computer were used for further calculations.

Tetracycline preparations. Tablets, dragees and hard- as well as soft-gelatine capsules were used for this investigation. An amount equivalent to 250 or 500 mg tetracycline hydrochloride was accurately weighed, treated with 30 ml methanol and diluted with water to give a concentration of 1 mg/ml. The sample solution was filtered through a Schleicher und Schüll filter paper no. 5893 and the first 20 ml were rejected. The remaining solution was used directly for HPLC measurements. The determination of TC and ETC was made with mobile phase I and a 1 : 10 diluted sample solution (100 μg/ml). The stock solution of 1 mg/ml and the mobile phase II were used for the separation of EATC and ATC traces.

Results and discussion

It could be demonstrated that the separation of all four compounds could be achieved with a reversed-phase C_{18} column loaded with about 20% chemically bonded organic phase, without further treating the column material to trialkylsilylate the remaining Si-OH groups, (Fig. 1a,b). Tetracyclines, which are virtually insoluble in hydrocarbon solvents, are retained by a hydrocarbon stationary phase. The k' values for ETC and TC are 1.69 and 2.5 (mobile phase I) and for TC, EATC and ATC are 0.55, 1.91 and 2.64 (mobile phase II), respectively. The effective plate heights for all four compounds were found to lie between 0.134 and 0.35 mm.

The effect of hydrogen-ion concentration has been reported to be slight on a ODS/TAS column and the key to the retention was attributed to the nature of the anion (Knox and Jurand, 1975). Nevertheless, the separation and elution of EATC and ATC on

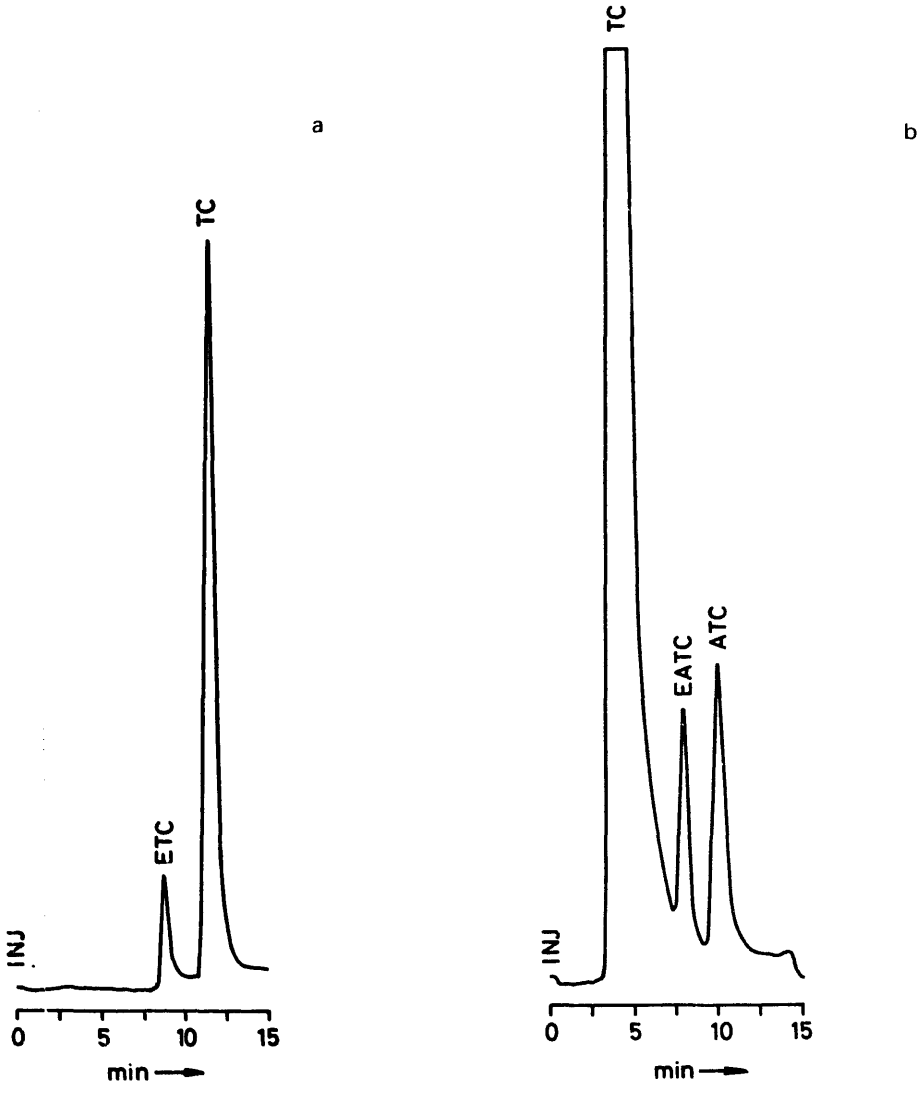

Fig. 1. a: Separation of epitetracycline (ETC) and tetracycline (TC) with mobile phase I. Flow rate: 1 ml/min; pressure: 800 psi. b: Separation of epianhydrotetracycline (EATC) and anhydrotetracycline (ATC) from tetracycline (TC) bulk with mobile phase II. Flow rate: 1 ml/min; pressure: 600 psi.

the ODS column used in this investigation was governed not only by the perchlorate anion but also by the acid concentration.

With a 0.2 M acid concentration sharp and symmetrical peaks were obtained for all four compounds. By further decreasing the perchloric acid concentration in the mobile phase (0.05–0.1 M) the components were either not eluted from the column on account of strong adsorption, or gave unsymmetrical peaks with tailing. The mobile phase I with 22% acetonitrile in 0.2 M $HClO_4$ still does not elute EATC and ATC from the column. An increase of the solvating agent acetonitrile in the mobile phase II to 37% finally enabled the elution of EATC and ATC after the TC peak (Fig. 1b). With this mobile phase ETC overlapped with the TC peak and therefore had to be determined with the

mobile phase I (Fig. 1a). The order of retention is in agreement with the polarity of tetracycline compounds, the most polar compounds EATC and ATC containing two aromatic rings with hydroxyl groups, only being eluted with an acetonitrile-rich mobile phase.

Formation of EATC and ATC due to partial degradation of ETC or TC in the strong acidic mobile phase was not noticed. Concentrated solutions of TC standard (2 mg/ml) injected into the liquid chromatograph did not show any traces of EATC or ATC. Six pharmaceutical preparations analyzed for these impurities contained 0.80–7.78% ETC, not detectable–0.8% EATC and not detectable–1.93% ATC.

There was a linear relationship between the concentration and peak area for all four compounds in the concentration range used of 20–100 μg/ml. The relative standard deviation of the method was found to lie between ±0.8 and 3.7%. The detection limit was about 5 μg/ml for an injection volume of 5 μl in the case of all four compounds.

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