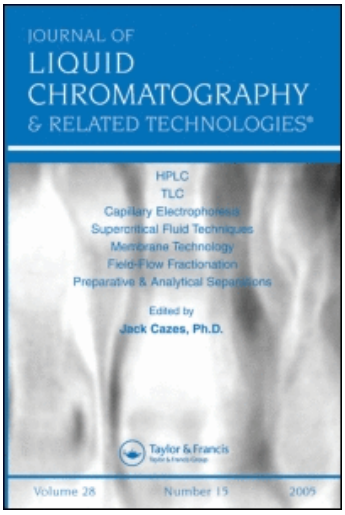

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SEPARATION OF OXYTETRACYCLINE AND ITS RELATED SUBSTANCES BY CAPILLARY ELECTROPHORESIS

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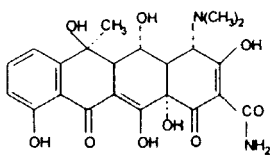
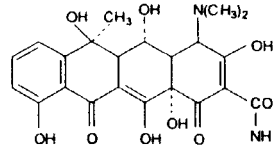
ABSTRACT

A method is described for separation of oxytetracycline and its related substances by capillary electrophoresis. Oxytetracycline is a broad spectrum antibiotic obtained by biosynthesis. Main related substances are 4-epioxytetracycline, α-apooxytetracycline, β-apooxytetracycline, anhydrooxytetracycline, 2-acetyl-2-decarboxamidooxytetracycline, tetracycline, 4-epitetracycline and terrinolide. Method development was undertaken with a mixture consisting of oxytetracycline and its related substances mentioned. Using an uncoated fused silica capillary, the influence of buffer pH and its concentration was systematically investigated. Non-ionic surfactant Triton X-100 was added to the running buffer for improving separation selectivity. Quantitative data are reported and compared with those of liquid chromatography and capillary zone electrophoresis.



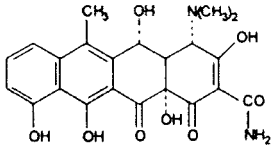
oxytetracycline (OTC)

epimerisation
pH 2 - 6



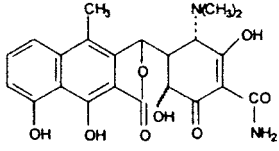
4-epioxytetracycline (EOTC)

acid



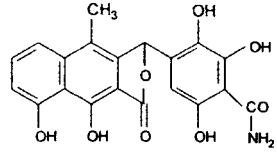
anhydrooxytetracycline (AOTC)

acid

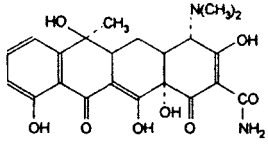


α- and β-apooxytetracycline
(α-APO and β-APO)

acid

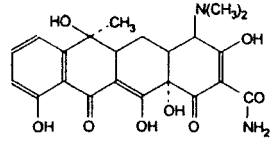


terrinolide (TL)

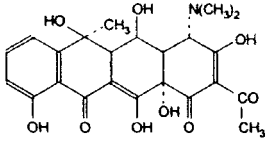


tetracycline (TC)

epimerisation
pH 2 - 6



4-epitetracycline (ETC)



2-acetyl-2-decarboxamidooxytetracycline
(ADOTC)

INTRODUCTION

Oxytetracycline (OTC) is a broad spectrum antibiotic obtained by biosynthesis. 2-Acetyl-2-decarboxamidooxytetracycline (ADOTC) and tetracycline (TC) are fermentation impurities of OTC. OTC can epimerise at position C-4, resulting in the formation of 4-epioxytetracycline (EOTC). The presence of a hydroxyl group at C-6 enables acid degradation, mainly occurring in OTC.HCl samples and forming anhydrooxytetracycline (AOTC). AOTC is quite unstable and rearranges in acid to two isomers, α-apooxytetracycline (α-APO) and β-apooxytetracycline (β-APO).[1] These can in turn react to form terrinolide (TL).[1] 4-Epitetracycline (ETC) can be present due to epimerisation of TC at position C-4.

The structures of OTC and its related substances are shown in Figure 1. Except for TC, the potential impurities are therapeutically inactive. Separation of OTC and its related substances is mainly performed by liquid chromatography (LC).[2,3]

However, capillary electrophoresis (CE) for analysis of OTC has also been reported in recent years. The separation of OTC from TC and chlortetracycline (CTC) with MECC[4] and the separation of OTC from six other tetracyclines[5] were described. A capillary zone electrophoresis (CZE) method to resolve OTC from its fermentation and degradation impurities was also investigated,[6] but an important impurity, ADOTC could not be separated from OTC in this system.

The method presented here is related to previous CE work concerning the analysis of tetracycline (TC),[7] doxycycline (DOX),[8] demeclocycline (DMCTC)[9] and minocycline (MC).[10]

In this paper, non-ionic surfactant Triton X-100 is employed to improve separation selectivity due to the interaction with the negatively charged analytes. This detergent moves at the speed of electroosmotic flow and has to be distinguished from the pseudostationary phase in electrokinetic chromatography which has its own electrophoretic mobility. Triton X-100 would also not allow the separation of neutral compounds.[11] The method enables the complete separation of OTC from its related substances AOTC, α-APO, β-APO, EOTC, TC, ETC, ADOTC and TL. The analysis time is shorter than with LC. Quantitative results are also compared with those of LC and CZE.

Figure 1 (left). Chemical structures of oxytetracycline and its related substances.

MATERIALS

Capillary electrophoresis was performed on Spectraphoresis 500 equipment (Thermo Separation Products, Fremont, CA, U.S.A.), coupled to a 3396 series II integrator (Hewlett Packard, Avondale, PA, U.S.A.). Tetracyclines were detected by UV absorption at 254 nm. Injection was done hydrodynamically for 4 seconds. pH measurements were performed on a Consort pH-meter (Turnhout, Belgium) using calibration buffers constituted according to the European Pharmacopoeia.[12] When necessary, the pH of running buffers was adjusted using 0.1 M HCl before making up to volume. Throughout the study, all samples were dissolved in running buffer to obtain better peak symmetry. To obtain repeatable migration times, it is advisable to wash the capillary each day consecutively with 0.1 M NaOH solution, 0.1 M phosphoric acid solution and 20 mM EDTA solution.

All reagents were of analytical grade (Merck, Darmstadt, Germany or Acros Chimica, Geel, Belgium). Oxytetracycline and its related substances were obtained from Acros Chimica. TL and AOTC were prepared by an existing method.[1] Small amounts of ADOTC were also prepared and isolated by a thin layer chromatographic method.[13] Fused silica capillary was from Polymicro Technologies (Phoenix, AZ. U.S.A.). Throughout the study, Milli-Q^{50} water was used (Millipore, Milford, MA, U.S.A.). All the solutions were filtered through 0.2μm nylon filters (Alltech, Laarne, Belgium).

RESULTS AND DISCUSSION

Method development was performed with an artifical mixture containing OTC, AOTC, α-APO, β-APO, EOTC, TC, ETC and TL. ADOTC was not available in sufficient quantities to include it in the mixture and therefore was only used in the final stage. Sodium carbonate was used as background electrolyte and 1 mM EDTA was added in all cases to prevent interaction of the tetracycline structure with metals through complexation. The following parameters were optimized consecutively: buffer pH, buffer concentration and Triton X-100 concentration. Influences of voltage and capillary temperature were also examined.

The pH is a very important parameter for improving selectivity in CE and small differences can cause the separation of closely related substances.[14] Therefore buffer pH was first optimized. This was performed with a sodium carbonate (20 mM) -EDTA (1 mM) buffer including 0.5 % (v/v) Triton X-100, at a voltage of 10 kV and a temperature of 10 °C. The variation of pH was

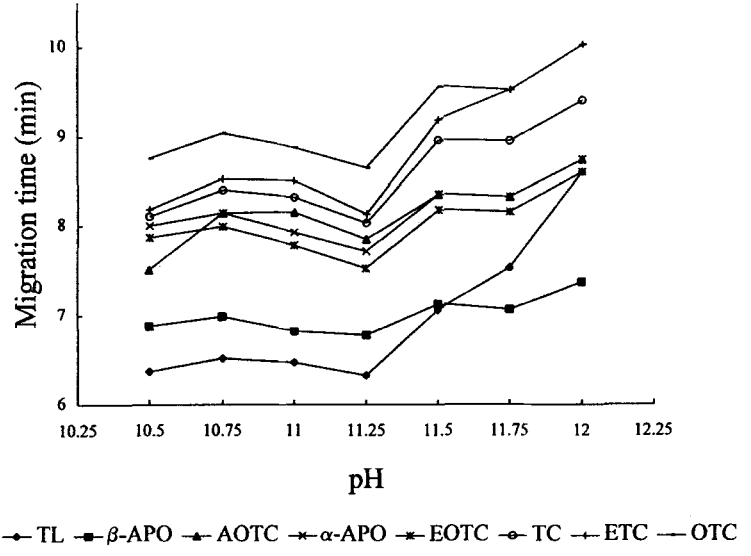


Figure 2. Influence of buffer pH on the separation of OTC and its related substances. Capillary: uncoated fused silica, L=44cm, l=38cm, ID=50μm; background electrolyte=sodium carbonate (20 mM)-EDTA (1mM) including 0.5 % (v/v) Triton X-100; temperature=10°C; voltage=10 kV.

restricted to the alkaline range to avoid sample adsorption on the capillary and epimerization of OTC. It was varied between 10.5 and 12.0 with steps of 0.25 pH unit. Since isoelectric points of OTC and its related substances are below the buffer pH, they are negatively charged and the electrophoretic mobility is opposite to the electroosmotic mobility. The changes of migration order and migration time are dependent on the sum of electrophoretic mobility of each solute and electroosmotic mobility. At one particular pH, the increase in the electrophoretic mobility of the negative ions might be dominant and result in an increase of migration time, and even migration order might change. However, at another pH, the increase of electroosmotic mobility can become dominant and result in a decrease of migration time which is unfavourable for resolution. Results are shown in Figure 2.

It was found that only at pH 11 and 11.25, separation of all eight substances could be obtained. A pH of 11 was retained because it gave a better resolution for the most critical separation TC/ETC. The order of migration

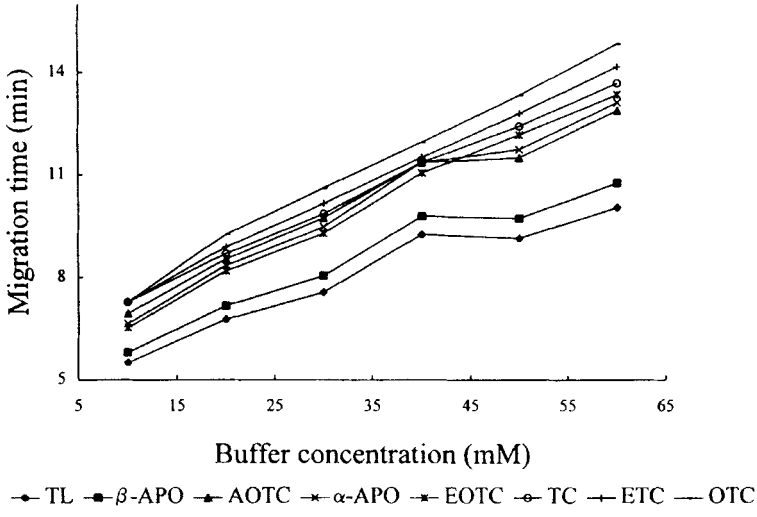


Figure 3. Influence of buffer concentration on the separation of OTC and its related substances. Capillary: uncoated fused silica, L=44cm, l=38cm, ID=50μm; background electrolyte=sodium carbonate (x mM)-EDTA (1mM) including 0.5 % (v/v) Triton X-100; pH=11.0; temperature=10°C; voltage=10 kV.

was: TL, β-APO, EOTC, α-APO, AOTC, TC, ETC and OTC. It should be pointed out that the buffer pH affected the order of migration in this system. At about pH 10.5, AOTC changed position around EOTC and α-APO. Above pH 11.5, the order of TL and β-APO also switched.

The next step was the optimization of buffer concentration. It has a main influence on electroosmotic flow and current produced in the capillary.[14] It was varied from 10 to 60 mM with steps of 10 mM, keeping other conditions constant (pH 11.0, 0.5 % Triton X-100, 10 kV, 10°C). Results are shown in Figure 3. All eight substances can be separated at 20 mM, 30 mM, 50mM and 60 mM. At 40 mM, their migration order changed. The explanation of the results is similar with the influence of the buffer pH. Under normal conditions, as the buffer concentration is increased, the electrophoretic and electroosmotic mobilities are both decreased. In this system, the decrease in the electroosmotic mobility with buffer concentration increase is dominant and results in an increase of migration time. 50 mM was finally chosen because it gave the best overall resolution.

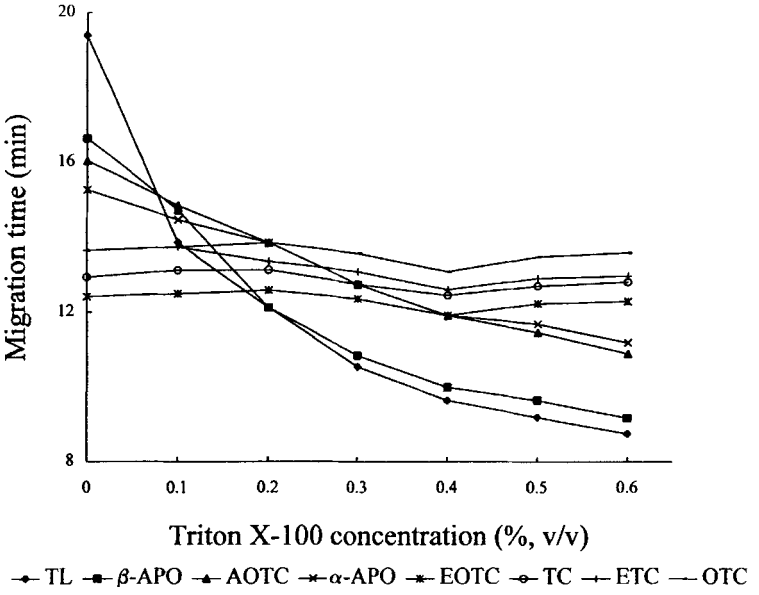


Figure 4. Influence of Triton X-100 concentration on the separation of OTC and its related substances. Capillary: uncoated fused silica, L=44cm, l=38cm, ID=50μm; background electrolyte=sodium carbonate (50 mM)-EDTA (1mM) including x % (v/v) Triton X-100; pH=11.0; temperature=10°C; voltage=10 kV.

The following step involved the optimization of Triton X-100 concentration in running buffer. It was varied from 0 to 0.6 % (v/v) with steps of 0.1 % (v/v). Only from 0.5 % (v/v) on, separation of all eight substances could be obtained. This concentration was finally retained because resolution between TC/ ETC decreased at 0.6 % (v/v). Results are shown in Figure 4 and can be explained by an interaction of the analytes with the alkyl chain of Triton X-100, possibly through hydrophobic interactions.

More hydrophobic compounds interact more strongly with the micelle and electrophoretic mobility decreases with increasing Triton X-100 concentration. So, migration time of the more hydrophobic compounds is shorter. This explanation can be confirmed by results of reversed phase LC[2,3] where elution order was almost opposite. In RPLC, the more hydrophobic compounds are retained longer. The selectivity of the system was markedly influenced by the Triton X-100 concentration, as migration order shows substantial changes.

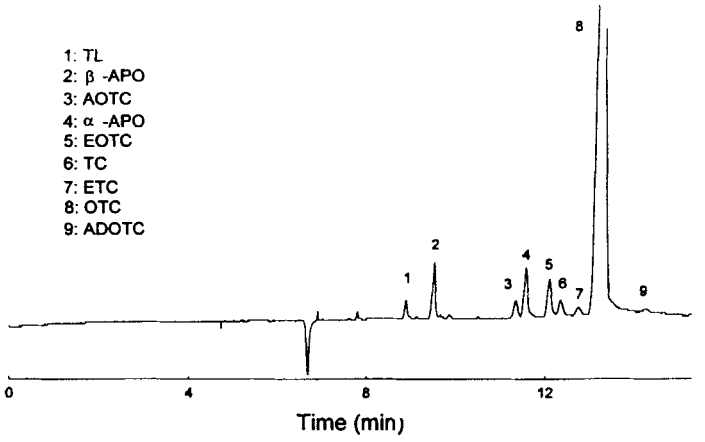


Figure 5. Electropherogram of oxytetracycline and its related substances. Capillary: uncoated fused silica, L=44cm, l=38cm, ID=50μm; background electrolyte=sodium carbonate (50 mM)-EDTA (1mM) including 0.5 % (v/v) Triton X-100; pH=11.0; temperature=10°C, voltage=10 kV.

It is shown from these results that in this system Triton X-100 concentration affected separation more than pH and buffer concentration. On the other hand, AOTC, TL and β-APO were affected more than other substances and it is difficult to explain this in detail from their structures.

In this stage, ADOTC was added to the sample. It was found that ADOTC can be separated from OTC with a resolution of 2.7. The order of migration under final optimal conditions is TL, β-APO, AOTC, α-APO, EOTC, TC, ETC, OTC and ADOTC. It is totally different from the CZE method,[6] where peak order was EOTC, TC, ETC, OTC, α-APO, AOTC, β-APO and TL.

Influence of capillary temperature and applied voltage on resolution was also investigated. It was found that separation became slightly worse with temperature and voltage increase, specifically for the critical pair TC/ETC. 10°C and 10kV were chosen as optimal conditions.

Figure 5 shows a typical electropherogram. Running buffer (excluding Triton X-100) was chosen as sample solvent, because it produced a better peak symmetry than 0.01 M HCl. A comparison with the performance of LC[2,3] shows that this method not only is better but also takes less time than LC. The latter analysis took over 30 min compared to 20 min for this method including

the washing procedure. Furthermore, the LC method needs a gradient elution. The repeatability was checked with the system shown in Figure 5 and using a spiked sample. The relative standard deviations (R.S.D.) (n=5) on corrected peak area (i.e. peak area divided by migration time) are given below, between brackets.

The sample used contained approximately 81.3 % w/w OTC (1.5 %), 5.0 % w/w of β-APO (5.7 %), , 4.4 % w/w of α-APO (3.4 %), 3.2 % w/w of EOTC (6.5 %), 1.4 % w/w of TC (13.5 %), 0.8 % w/w of ETC (8.2 %), 0.3 % w/w of ADOTC (22.7 %), 1.1 % w/w of TL and 2.5 % w/w of AOTC. The R.S.D.s of TL and AOTC are not given, because these compounds are too unstable in solution. Similar R.S.D.'s could be obtained with CZE (1.0 %, n=8).[6] As is generally the case, the R.S.D. for OTC is higher than with LC, where an R.S.D. value of 0.3 % was obtained for OTC.[2] The limit of detection (S/N = 3) was 0.05 % and the limit of quantification was 0.1 % for OTC (n=7, R.S.D.=17 %) with respect to the peak obtained with a sample solution containing 1.0 mg/mL OTC. The loadability of the system was also investigated. The fronting of the OTC peak increased with the loading. For the usually injected amount of approximately 7 ng (1 mg/mL, 4 sec), the peak symmetry factor was 0.7 and the separation of ETC and OTC was not affected until a solution containing 1.5 mg/mL of OTC was injected. The following calibration line was obtained for OTC: Y= 1396+140107 X, with Y= corrected peak area, X= concentration of the analysed solution in mg/mL, r= 0.9994, Sy,x (standard error of y-estimate)=2702, investigated range =0.25-1.75 mg/mL, 7 points (n=2).

As a conclusion it can be stated that complete separation of OTC and its related substances was achieved by CE using a non-ionic surfactant. It offers the advantages of speed over LC and slightly better selectivity, but LC performs better in quantitative analysis.

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