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Purity control of oxytetracycline by capillary electrophoresis

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Abstract

The applicability of capillary electrophoresis for the purity control of oxytetracycline (OTC) was investigated. OTC is a broad-spectrum antibiotic belonging to the group of the tetracyclines. Several related substances can be present due to fermentation or degradation, such as 4-epioxytetracycline, α-apooxytetracycline, β-apooxytetracycline, anhydrooxytetracycline, 2-acetyl-2-decarboxamidooxytetracycline, tetracycline and 4-epitetracycline. Using fused-silica capillaries, the influence of buffer type, buffer pH and buffer concentration were investigated. In all cases 1 m*M* EDTA was added to prevent metal-ion complexation. The influence of the buffer counter-ion type was examined. Consequently, some instrumental parameters were changed such as capillary length and diameter as well as capillary temperature and applied voltage. The following method is finally proposed: fused-silica capillary, *l* (effective length)=38 cm, *L* (total length)=44 cm, 50 μm I.D.; buffer, sodium carbonate 20 m*M*–EDTA 1 m*M*, pH 11.25; voltage, 10 kV; temperature, 10°C. Linearity, limit of detection and limit of quantitation were determined as well as the relative standard deviations for all the analytes involved. This method is less selective then existing liquid chromatographic methods but it may be used as a complementary tool in purity control and stability studies.

Keywords: Capillary electrophoresis; Oxytetracyline; Antibiotics

1. Introduction

Oxytetracycline (OTC) is a broad-spectrum antibiotic of the family of the tetracyclines. It is produced through fermentation by *Streptomyces rimosus*. During this process some side products can be formed, such as 2-acetyl-2-decarboxamidooxytetracycline (ADOTC) [1,2] and tetracycline (TC) [3]. Manufacturing OTC as the hydrochloride salt brings it into contact with acid which may cause conversion of OTC to 4-epioxytetracycline (EOTC) through an equilibrium reaction [4] and to anhydrooxytetracycline (AOTC) by irreversible dehydration. AOTC is quite unstable and readily transforms into α-apooxytetracycline (α-APO) and β-apooxytetracycline (β-APO) [4]. These can in turn react to form terrinolide (TL) [4]. Contact with acid can also convert TC to 4-epitetracycline (ETC) [5], and thus the presence of ETC has to be taken into account. The structures of the compounds studied are shown in Fig. 1. The pK_a values of OTC are 3.27, 7.32, 9.11 and 10.7 [6,7].

The present paper explores the applicability of capillary electrophoresis (CE) for the purity control of OTC. A non-ionic micellar system using Triton X-100 in a phosphate buffer pH 2.2 was described

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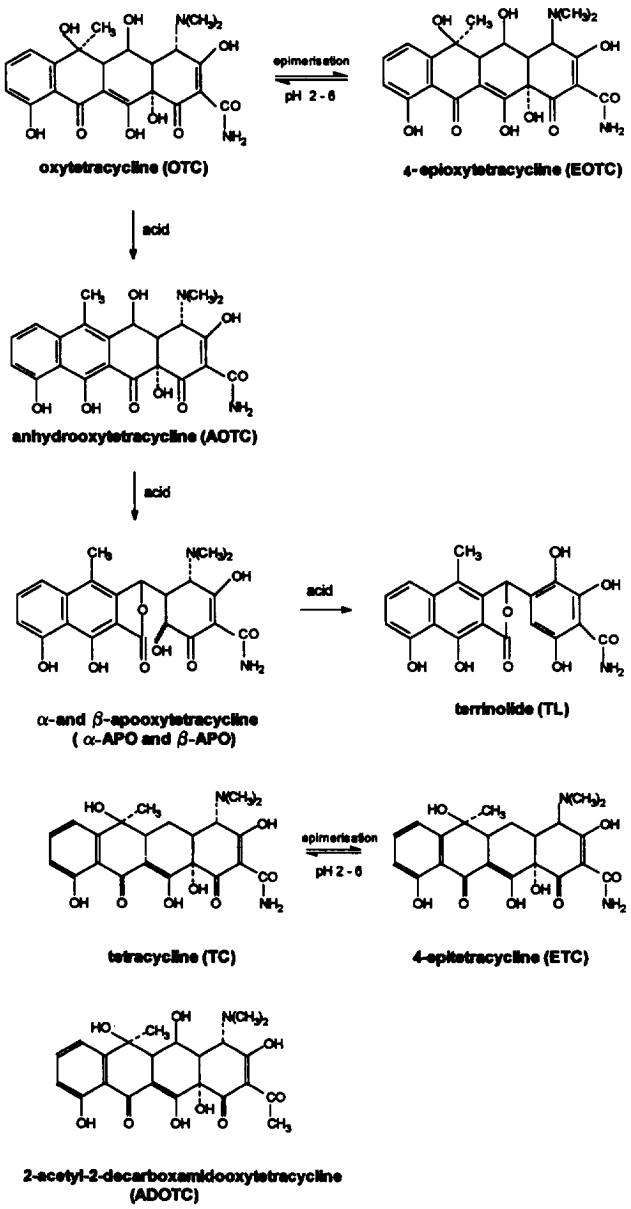

Fig. 1. Structures of OTC and its potential impurities.

for the separation of OTC from TC and chlortetracycline (CTC) in a time period of 20 min [8]. Other workers claimed that a 0.0043 *M* phosphate buffer at pH 7.5 satisfactorily separated OTC from six other tetracyclines [9]. However, a CE method to resolve OTC from its fermentation and degradation impurities has not been previously described.

2. Experimental

Capillary electrophoresis was performed on Spectraphoresis 500 equipment (Thermo Separation Products, Fremont, CA, USA), coupled to a 3396 Series II integrator (Hewlett-Packard, Avondale, PA, USA) which reported peak areas. Tetracyclines were de-

tected by UV absorption at 254 nm. Injection was done hydrodynamically. Fused-silica capillaries were from Polymicro Technologies (Phoenix, AZ, USA). Throughout the study, Milli-Q50 water was used (Millipore, Milford, MA, USA). All the solutions were filtered through 0.2-μm nylon filters (Alltech, Laarne, Belgium). pH measurements were performed on a Consort pH meter (Turnhout, Belgium) using calibration buffers constituted according to the European Pharmacopoeia [10]. Reagents were of analytical grade (Merck, Darmstadt, Germany, or Acros Chimica, Geel, Belgium). When necessary, the pH of buffers was adjusted using either HCl or NaOH before making up to volume. Oxytetracycline and its related substances were obtained from Acros Chimica. Electrophoretic parameters were determined using mixtures containing approximately equal amounts of OTC, EOTC, TC, ETC, α-APO and β-APO in a concentration of $\pm$0.004% w/v each. AOTC was not added because it is too unstable in solution. ADOTC was not available in sufficient quantities to be included in the mixture and therefore was only used in the final stage. The method was also applied to mixtures resembling real commercial samples, i.e. containing mainly OTC. To obtain repeatable migration times, it is advisable to wash the capillary after each analysis consecutively with 0.1 *M* NaOH, 0.1 *M* phosphoric acid and 20 m*M* EDTA.

3. Results and discussion

In view of reports on the influence of the buffer on selectivity [11], it was found mandatory to first compare the performance of different types of background electrolytes for the analysis of OTC. Five buffers were tested each in a concentration of 100 m*M* and at pH 8.6: sodium phosphate, Tris, sodium tetraborate, sodium carbonate and EDTA. The former four buffers also contained 1 m*M* EDTA to prevent the well-known interference of tetracyclines with metal ions. With the first two buffers several components of the mixture could not be separated and sodium tetraborate had a broadening effect on the peaks. With sodium carbonate or EDTA the test mixture was well separated, but subsequent experiments were nevertheless performed with sodium carbonate because of shorter migration times (data not shown). The next parameter to be investigated was pH. Since small differences in pK_a can cause the separation of closely related substances, this parameter is critical for method development. Experiments were done using sodium carbonate (20 m*M*)–EDTA (1 m*M*) buffer on a fused-silica capillary of 70 cm length and 75 μm I.D. The applied voltage was 12 kV and the temperature 15°C. The pH was varied between 8 and 12 with steps of at least half a pH unit. Especially separation of TC and ETC was difficult but could be obtained at pH 11.25. Resolution is shown in Fig. 2, which also shows that pH adjustment of the buffer should be performed very carefully since at pH 11.5 separation between EOTC and TC is lost.

The concentration of the buffer has a major influence on the electro-osmotic flow and the current produced in the capillary. It was therefore varied between 10 and 50 m*M* keeping the EDTA concentration at 1 m*M*. Table 1 shows the obtained resolutions, the migration time of the last eluted compound and the current in the system. Taking into account separation, speed and current, the initially chosen concentration of 20 m*M* sodium carbonate was maintained.

Resolution versus capillary temperature is shown in Fig. 3. An important reason for choosing 20°C, apart from the good separation and the proximity to room temperature, is the fact that AOTC does not

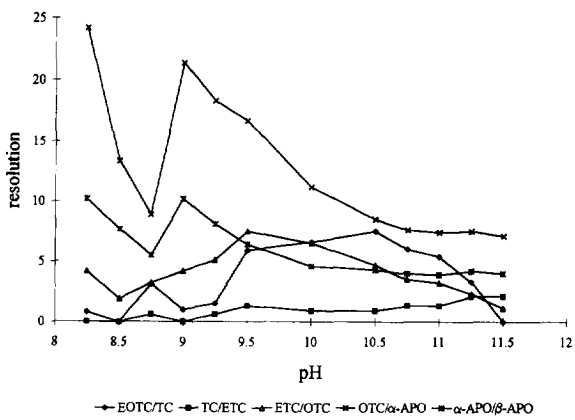

Fig. 2. Resolution between components of the OTC test mixture as a function of pH. Capillary: fused silica, L=70 cm, l=62 cm, I.D.=75 μm; background electrolyte, sodium carbonate (20 m*M*)–EDTA (1 m*M*); voltage, 12 kV; temperature, 15°C.

Table 1
Resolution, current and total analysis time as a function of concentration of the sodium carbonate buffer

Buffer concentration (m*M*)	Resolution					Current (μA)	Analysis time (min)
	EOTC/TC	TC/ETC	ETC/OTC	OTC/α-APO	α-APO/β-APO		
10	2.5	1.0	1.5	4.9	2.6	17	20
20	3.3	2.1	2.3	7.5	4.2	30	29
30	3.1	2.7	2.6	8.7	5.1	44	35
40	2.7	3.2	2.8	9.8	6.0	58	39
50	2.4	4.1	3.2	11.8	7.4	70	52

Capillary: fused silica, L=70 cm, l=62 cm, I.D.=75 μm; background electrolyte, sodium carbonate (x m*M*)–EDTA (1 m*M*), pH 11.25; voltage, 12 kV; temperature, 15°C.

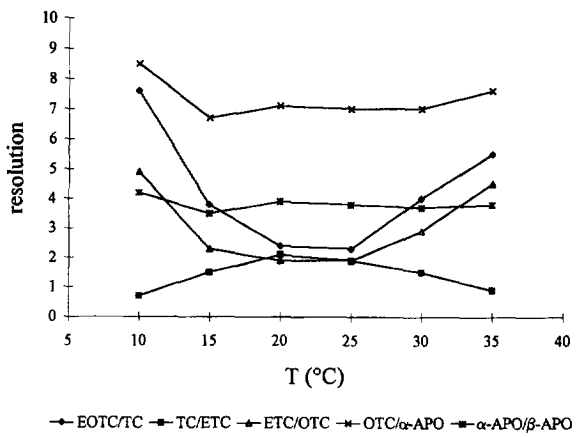

Fig. 3. Resolution between components of the OTC test mixture as a function of capillary temperature. Capillary: fused silica, L=70 cm, l=62 cm, I.D.=75 μm; background electrolyte, sodium carbonate (20 m*M*)–EDTA (1 m*M*), pH 11.25; voltage, 12 kV.

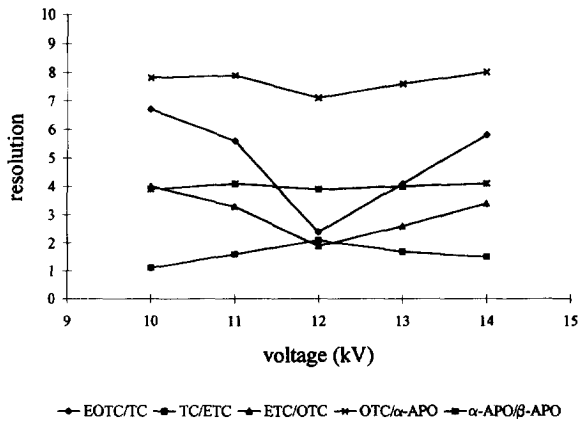

Fig. 4. Resolution between components of the OTC test mixture as a function of applied voltage. Capillary: fused silica, L=70 cm, l=62 cm, I.D.=75 μm; background electrolyte, sodium carbonate (20 m*M*)–EDTA (1 m*M*), pH 11.25; temperature, 20°C.

co-migrate with β-APO at this temperature. Selectivity is thus influenced quite strongly by temperature. The latter can have various effects on separation in CE in terms of Joule heat developed, changes in electro-osmotic flow and impact on chemical equilibria taking place in the capillary [12]. The change in selectivity observed here is most probably related to the fact that pK_a and mobility differ for the different constituents of the sample with respect to temperature [13]. Selectivity differences might also be related to the buffer undergoing pH changes due to temperature changes [12,13]. The applied voltage was also found to influence selectivity (see Fig. 4) and 12 kV was selected. Since the

Table 2
Resolution, current and total analysis time as a function of the buffer counter-ion

Buffer counter ion	Resolution					Current (μA)	Analysis time (min)
	EOTC/TC	TC/ETC	ETC/OTC	OTC/α-APO	α-APO/β-APO		
Sodium	2.4	2.1	1.9	7.1	3.9	31	22
Potassium	3.7	1.6	1.9	6.3	3.9	38	21
Ammonium	3.8	1.8	1.6	7.2	4.7	45	23

Capillary: fused silica, L=70 cm, l=62 cm, I.D.=75 μm; background electrolyte, sodium, potassium or ammonium carbonate (20 m*M*)–EDTA (1 m*M*), pH 11.25; voltage, 12 kV; temperature, 20°C.

Table 3
Resolution, current and total analysis time as a function of capillary length and internal diameter

	Resolution					Current (μA)	Analysis time (min)
	EOTC/TC	TC/ETC	ETC/OTC	OTC/α-APO	α-APO/β-APO		
L=70 cm							
I.D.=30 μm	1.2	1.6	1.0	4.5	3.3	4	22
I.D.=50 μm	1.2	2.4	1.5	7.1	4.0	12	22
I.D.=75 μm	2.4	2.1	1.9	7.1	3.9	31	22
L=44 cm							
I.D.=30 μm	–	1.1	–	2.7	1.8	7	11
I.D.=50 μm	–	1.2	0.9	3.6	2.3	20	11
I.D.=75 μm	–	1.1	–	3.2	1.7	48	11

Capillary: fused silica; background electrolyte=sodium carbonate (20 m*M*)–EDTA (1 m*M*), pH 11.25; voltage=12 kV; temperature=20°C.

TC/ETC/OTC separation was still rather critical, the influence of the buffer counter-ion was investigated. Results are summarized in Table 2. Current was most favourable with sodium carbonate, as well as selectivity.

At this stage it was found opportune to try to shorten the analysis time, which amounted up to 20 min. Therefore studies were undertaken with shorter capillaries of which the internal diameter was also varied. The influence of these parameters on resolution and current values is shown in Table 3. Care was taken to obtain electropherograms in which the corrected peak areas were comparable for all dimensions of the capillary. This could be obtained by adjusting sample concentrations while keeping the injection time constant. It can be concluded that a diameter of 50 μm is more favourable in view of the current and separations obtained with both lengths of the capillary. Since resolutions are not deteriorating too much with diminishing length of the capillary and since a considerable time gain can be achieved, it was attempted to slightly adjust the applied voltage and capillary temperature for the short capillary. This yielded a combination of 10 kV and 10°C (data not shown). Fig. 5 shows electropherograms of a mixture of OTC and its related substances analyzed on the long and short capillary. In both cases the migration time of ADOTC was found to be the same as that of OTC. This is a disadvantage of CE vs. liquid chromatography (LC), the selectivity of which does allow for the separation of ADOTC [14–16]. A typical LC chromatogram is shown in Fig. 6. A comparison with the LC performance shows that the most apparent difference is the speed of CE with respect to LC. The latter analysis takes 45 min compared to 30 min for CE including the washing procedure.

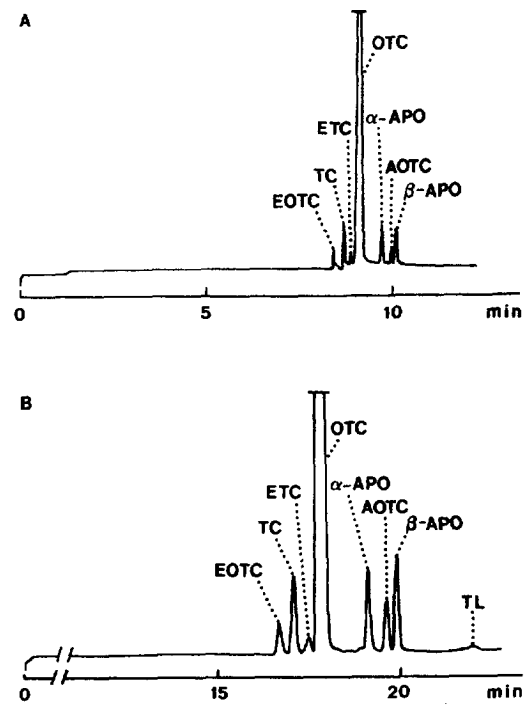

Fig. 5. Electropherogram of a mixture of OTC and its related substances. (A) Capillary: fused silica, L=44 cm, l=38 cm, I.D.=50 μm; background electrolyte, sodium carbonate (20 m*M*)–EDTA (1 m*M*), pH 11.25; temperature, 10°C; voltage, 10 kV. (B) Capillary: fused silica, L=70 cm, l=62 cm, I.D.=75 μm; background electrolyte, sodium carbonate (20 m*M*)–EDTA (1 m*M*) pH 11.25; temperature, 20°C; voltage, 12 kV.

Table 4
Quantitative data for CE

	EOTC	TC	ETC	OTC	α-APO	AOTC	β-APO
Normalized peak area percentage	1.77	4.77	1.20	81.16	3.92	2.46	4.72
R.S.D. (%)							
Method 1 (n=6)	8.0	5.9	18.2	4.1	13.6	28.0	5.0
Method 2 (n=8)	2.4	0.8	1.3	1.0	2.8	14.0	1.1
LOD (S/N=3)				10^{-5}% w/v			
Method 2				0.5 pg			
R.S.D.=42.7% (n=3)				0.02%			
LOQ (S/N=27)				10^{-3}% w/v			
Method 2				50 pg			
R.S.D.=15.6% (n=5)				2.3%			
Linearity (y=corrected area; x=OTC concentration in mg/ml)							
Method 1	range=0.5–1.8 mg/ml	r=0.9982	$y=-1516+75980x$				
Method 2	range=0.14–0.23 mg/ml	r=0.9969	$y=-7011+424411x$				

Method 1: fused-silica capillary, L=44 cm, l=38 cm, I.D.=50 μm; background electrolyte, sodium carbonate (20 mM)–EDTA (1 mM), pH 11.25; temperature, 10°C; voltage, 10 kV. Method 2: fused-silica capillary, L=70 cm, l=62 cm, I.D.=75 μm; background electrolyte, sodium carbonate (20 mM)–EDTA (1 mM), pH 11.25; temperature, 20°C; voltage, 12 kV.

Table 4 contains some quantitative data for CE. Relative standard deviations on corrected peak areas are much better on the wider capillary. Since corrected peak areas were designed to be comparable for both capillaries, the effect of a higher pathlength cannot explain the better R.S.D. values. It is believed that the larger volume of injection together with higher resolutions obtained on the wider capillary produce better repeatability. Quantitatively, LC performs better than CE, as is generally the case. The R.S.D. value for OTC was indeed 0.3% using LC. LOD and LOQ values were determined on the long capillary and are given in concentration as well as mass units. The percentage values mentioned represent the percentage of the amount injected in an assay and give an idea of the extent to which the method can still detect and quantitate impurities which are present in low amounts. Linearity was proven for OTC with both methods.

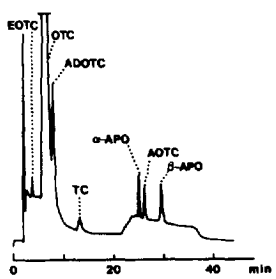

Fig. 6. LC chromatogram of a mixture of oxytetracycline and its related substances, analyzed according to Ref. [16] but doubling the amount of tetrabutylammonium sulphate and using a stationary phase with 5 μm particle diameter instead of the 8 or 10 μm prescribed. Column: styrene–divinylbenzene copolymer 5 μm; mobile phase: 2-methyl-2-propanol–0.33 M phosphate buffer pH 7.5–1% w/v tetrabutylammonium sulphate pH 7.5–0.04% w/v sodium edetate pH 7.5–water [5.1 (first 15 min) and 7.9 (following 15 min):6:10:10:68.9 (first 15 min) and 66.1 (following 15 min), w/v/v/v/v]; flow-rate, 1.0 ml/min; temperature, 60°C; detection, UV at 254 nm.

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