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Determination of impurities in tetracycline hydrochloride by non-aqueous capillary electrophoresis

Jette Tjørnelund*, Steen Honoré Hansen

Department of Analytical and Pharmaceutical Chemistry, The Royal Danish School of Pharmacy, Universitetsparken 2, 2100 Copenhagen, Denmark

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Abstract

A highly selective method for quantitative determination of impurities in tetracycline antibiotics based on non-aqueous capillary electrophoresis has been developed. In tetracycline hydrochloride the degradation products 4-epitetracycline, anhydrotetracycline and 4-epianhydrotetracycline can be determined with limits of detection corresponding to 0.06%, 0.04% and 0.02% of the drug substance, respectively. The relative standard deviations were about 4% at the 0.1% level of impurity in tetracycline hydrochloride. Furthermore, it is possible to detect desmethyltetracycline as well as a number of unknown impurities within 10 min. The separation of tetracycline, oxytetracycline, doxycycline and chlortetracycline by non-aqueous capillary electrophoresis is also demonstrated. The nature of the solvents and electrolytes in the electrophoresis medium exhibit a major influence on the separation selectivity.

Solvents such as methanol and acetonitrile are more volatile than water and it is demonstrated that evaporation of the solvent from sample as well as from the electrophoresis medium may cause severe problems in some capillary electrophoresis instruments.

Keywords: Pharmaceutical analysis; Tetracyclines; Antibiotics

1. Introduction

Tetracyclines are one of the most important groups of the broad-spectrum antibiotics. Tetracycline (TC) itself is an unstable compound [1]. Below pH 2 dehydration of TC is the predominant reaction. This results in the formation of anhydrotetracycline (ATC). At pH 2–6 TC undergoes a reversible epimerization reaction at C-4 leading to the formation of an equilibrium mixture of TC and 4-epitetracycline (ETC). ATC also undergoes epimerization resulting in the formation of 4-epianhydrotetracycline (EATC). In neutral and weakly basic aqueous solution oxidation of tetracycline is observed. The product formed in alkaline solution has been shown to be isotetracycline [1]. Futhermore, other related compounds, 2-acetyl-2-decarboxamidotetracycline and desmethyltetracycline (DTC) has been described [2] as potential impurities in TC.

The degradation of tetracycline strongly influences the pharmacological activity. The epimerization of TC is known to reduce the antibiotic activity to 2–5% [3] of that of TC and EATC has been implicated in toxic manifestations such as renal dysfunction and the reversible Fanconi-type syndrome [4–6].

*Corresponding author.

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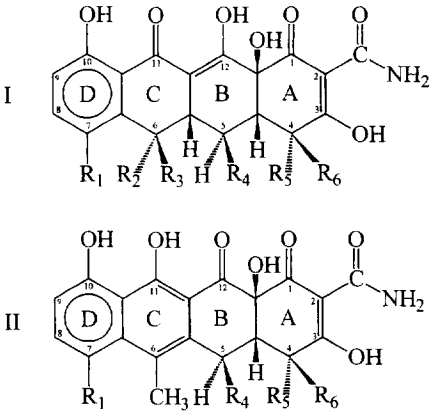
The separation of the tetracyclines and their impurities have been a challenge to many chromatographers when using HPLC [7–11]. The peak shapes of these compounds often tend to be characterized by tailing and low efficiency due to interaction with the residual silanol groups on silica-based packing materials. In order to overcome this problem adjustment of pH of the mobile phase as well as addition of complexing agents such as citric acid, oxalic acid or disodium ethylenediaminetetraacetate (EDTA) have been common approaches [7–9]. Polymer-based columns have shown to be very useful for separation of tetracycline and related compounds [9–11]. However, relatively long times of analysis are often needed in order to separate TC and potential impurities.

In the last few years reports on separation of tetracyclines by aqueous capillary electrophoresis (CE) have appeared [12–14]. Taveres and McGuffin [12] used a sodium phosphate buffer pH 7.5 to separate tetracycline and its degradation products in 7 min. However, it was difficult to completely separate mixtures of commercially available tetracyclines. Croubles et al. [13] used micellar electrokinetic chromatography at pH 2.2 for similar separations in 24 min and Zhang et al. [14] found that it was necessary to carefully control the concentration of the EDTA as an additive in 0.02 *M* phosphate buffer pH 3.9 in order to obtain the separation of tetracyclines and its degradation products. Recently, successful applications of non-aqueous capillary electrophoresis have been reported [15–21]. It was shown that selectivities, that were very difficult to obtain in aqueous buffers even when using MEKC or complexing agents were easily obtained when using non-aqueous systems [18,20].

In this paper a selective non-aqueous CE system for the determination of impurities in TC hydrochloride raw materials is presented. It is demonstrated that non-aqueous CE is a valuable supplement to HPLC in order to reveal unknown impurities. Furthermore, non-aqueous CE of oxytetracycline, doxycycline and chlortetracycline is described. The structures of the tetracyclines are shown in Fig. 1.



Compound	Structure	Code	R_1	R_2	R_3	R_4	R_5	R_6
Tetracycline	I	TC	H	OH	CH_3	H	H	$N(CH_3)_2$
Chlortetracycline	I	CTC	Cl	OH	CH_3	H	H	$N(CH_3)_2$
Oxytetracycline	I	OTC	H	OH	CH_3	OH	H	$N(CH_3)_2$
Doxycycline	I	DC	H	H	CH_3	OH	H	$N(CH_3)_2$
Desmethyltetracycline	I	DTC	H	OH	H	H	H	$N(CH_3)_2$
4-Epitetracycline	I	ETC	H	OH	CH_3	H	$N(CH_3)_2$	H
Anhydrotetracycline	II	ATC	H	-	-	H	H	$N(CH_3)_2$
4-Epianhydro-tetracycline	II	EATC	H	-	-	H	$N(CH_3)_2$	H

Fig. 1. The chemical structures of some tetracycline antibiotics and related substances.

2. Experimental

2.1. Chemicals

Dimethyl sulphoxide (DMSO) was obtained from Merck (Darmstadt, Germany). Ammonium acetate, ammonium formate, methanesulphonic acid and N-methyl formamide (NMF) were obtained from Aldrich (Steinheim, Germany). N,N-Dimethylacetamide (DMA) was obtained from Fluka (Buchs, Switzerland). Acetic acid and N,N-dimethylformamide (DMF) were obtained from Riedel-de Häen (Seelze, Germany). Citric acid monohydrate was purchased from Ferak (Berlin, Germany) and dipotassium EDTA dihydrate from BDH (Poole, UK). Lithium chloride was obtained from Sigma (St. Louis, MO, USA). The used methanol (MeOH) and acetonitrile (MeCN) were of HPLC grade and all chemicals were used without further purification.

TC hydrochloride, ETC hydrochloride, ATC hydrochloride, EATC hydrochloride, oxytetracycline (OTC) hydrochloride, chlortetracycline (CTC) hy-

drochloride and doxycycline (DC) hyclat were gifts from Nycomed DAK (Roskilde, Denmark) and from Dumex (Copenhagen, Denmark). DTC was a donation from Prof. J. Hoogmartens (Katholieke Universiteit, Leuven, Belgium).

2.2. Apparatus

An HP^{3D} CE system (Hewlett-Packard, Waldbronn, Germany) equipped with an on-column diode array detector (DAD) was used unless otherwise stated. A detection wavelength of 254 nm was used for all samples unless otherwise stated. The separation was performed in a fused-silica capillary (64 cm×50 μm I.D.; 55.5 cm to the detector) (Polymicro Technologies, Phoenix, AZ, USA). The capillary was thermostated to 25°C by air unless otherwise stated. Samples were kept at ambient temperature in the autosampler and injected by applying a pressure of 50 mbar (5 kPa) for 1 s unless otherwise stated. The injection of the sample was followed by injection of electrophoresis medium at a pressure of 50 mbar for 3 s. A voltage of 30 kV was applied during analysis unless otherwise stated.

Prior to use the capillaries were rinsed with 1 *M* sodium hydroxide for 60 min, 0.1 *M* sodium hydroxide for 20 min, distilled water for 20 min, MeOH for 20 min and 15 min with the final electrophoresis medium. Between analysis capillaries were flushed for 2 min with the electrophoresis medium. The electrophoresis medium was used for up to six runs unless otherwise stated.

2.3. Sample preparation

Samples were dissolved in the solvent or the solvent mixture corresponding to the electrophoresis medium. For quantitative measurements all samples were freshly prepared and protected against day light.

2.4. Suitability of the apparatus

The electrophoresis medium was used for fifteen successive runs of 5 mg/ml TC in MeOH–MeCN–DMF (45:49:6, v/v) with 1-h intervals in order to test the robustness of the method. The same sample vials were used during the runs in order to study the extent of evaporation from sample vials. The performance of the method using three different CE systems from Hewlett-Packard (HP^{3D}), Waters (Quanta 4000) (Milford, MA, USA) and Beckman (P/ACE 5010) (Fullerton, CA, USA), respectively, were compared.

The HP^{3D} CE system was used with a sample vial containing 400 μl sample solution and two buffer vials each filled with 400 μl electrophoresis medium. Injection was performed by pressure for 1 s at 50 mbar (5 kPa). Capillary: 64 cm×50 μm I.D.; 55 cm to the detector. The Beckman P/ACE 5010 CE system was used with a sample vial containing 350 μl sample solution and two buffer vials each filled with 4.4 ml electrophoresis medium. Injection: high pressure for 1 s at 0.5 p.s.i. (3.5 kPa). Capillary: 57 cm×50 μm I.D.; 50 cm to the detector. The Waters Quanta 4000 instrument was used with a sample vial containing 400 μl sample and buffer vials containing 17 ml of electrophoresis medium. Capillary: 57 cm×50 μm I.D.; 49.5 cm to the detector. Hydrostatic injection performed by raising the sample vial 10 cm (approx. 1.0 kPa) for 5 s.

3. Results and discussion

In order to explore the possibilities for obtaining resolution of the tetracyclines in non-aqueous CE, the influence of the nature of organic solvent, the choice of electrolytes and the temperature surrounding the capillary were investigated.

3.1. Choice of electrolyte

Electrolytes resulting in acidic properties in non-aqueous media were chosen in order to keep the tetracyclines as cations. Methanesulphonic acid was found to give a better resolution of tetracycline and its impurities than acetic acid and formic acid used in concentrations up to 1 *M*. Methanesulphonic acid is a very strong acid and tetracycline degrades to form ATC in acidic media. However, there are no indications of degradation of TC during the electrophoretic run as the peaks corresponding to TC and ATC do neither front nor tail in the final electrophoretic system.

The choice of the supporting electrolyte had a

great impact on the peak shapes obtained (Fig. 2A–F). This was probably partly due to the various mobilities of the electrolytes in the electrophoretic medium compared to the sample solutes, but also the solvating power of the solvents may play a role. A very efficient system was obtained using lithium chloride as electrolyte (Fig. 2E). However, the current was unstable probably due to electrode processes (electrolysis), and the system was not used for further studies. Some additives like citric acid and EDTA may complex metal ions present as impurities in the buffer substances or adsorbed to the silica surface resulting in more symmetrical peaks. However, EDTA did not improve peak shapes in the electrophoresis medium investigated (Fig. 2F).

3.2. Organic solvent

The nature of the organic solvent or solvent mixtures used for the electrophoresis medium may have a strong influence on the separation selectivity (Fig. 3 and Fig. 4). The separation of TC, OTC, CTC and DC is improved by adding small amounts of DMF to a mixture of MeOH–MeCN (48:52, v/v) while keeping the electrolytes constant (25 m*M* ammonium acetate, 10 m*M* citric acid and 118 m*M* methanesulphonic acid) as shown in Fig. 3. The electroosmotic flow (EOF) is decreasing with increasing concentrations of DMF in the solvent mixture. It has been proposed that an increased viscosity leads to a decrease in the EOF and a reduction of the efficiency unless this is compensated for by an increase in the dielectric constant [21]. MeOH, MeCN and DMF have similar dielectric constants but DMF has a higher viscosity than MeOH and MeCN and slows down the electroosmotic flow-rate. Combined with proteolytic and solubilization properties, different from the properties of MeOH and MeCN, DMF improves the resolution of the tetracyclines in concentrations up to 6%. The effect of adding DMA, NMF or DMSO to the electrophoresis medium was also studied. All the mentioned additives improved the resolution of the tetracycline antibiotics but DMF was a slightly better choice.

The changes in selectivity obtained by various mixtures of MeCN and MeOH keeping the electrolytes (25 m*M* ammonium acetate, 10 m*M* citric acid and 118 m*M* methanesulphonic acid) as well as the percentage of DMF (6%) constant in the electrophoresis medium is shown in Fig. 4. MeCN having the lowest viscosity improves the efficiency of the system whereas MeOH reduces the EOF but improves the selectivity between the tetracyclines. Moreover, the presence of MeOH in the medium makes it possible to dissolve sufficient amounts of ammonium acetate and citric acid in the electrophoresis medium.

3.3. Temperature

The effect of the temperature surrounding the capillary on the separation of a mixture of TC, OTC, CTC and DC was investigated. Only minor differences in selectivity were observed when running at 10, 25 or 40°C. As expected the migration times decreased with increasing temperature due to a decrease in the viscosity of the electrophoresis medium. The efficiency of the system was not affected by the changes in temperature.

4. Application

A highly selective method was developed and used for the determination of impurities in tetracycline hydrochloride. The structures of TC and its potential impurities ETC, DTC, ATC and EATC are shown in Fig. 1. TC and the impurities are completely separated with the exception of EATC and DTC that are only partly separated but quantitation of both compounds is still possible. Furthermore, a number of unknown impurities are detected in the electropherograms. The obtained selectivities are comparable or even better than the selectivities obtained in aqueous CE [12–14] and in HPLC [9–11]. Furthermore, the analysis time is shorter than the analysis time needed for separations by HPLC [9–11]. An electropherogram of 5.0 mg/ml TC is shown in Fig. 5.

4.1. Repeatability and linearity

The repeatability and the linearity of the method using the HP^{3D} instrument are shown in Table 1. The repeatability and the linearity obtained using non-

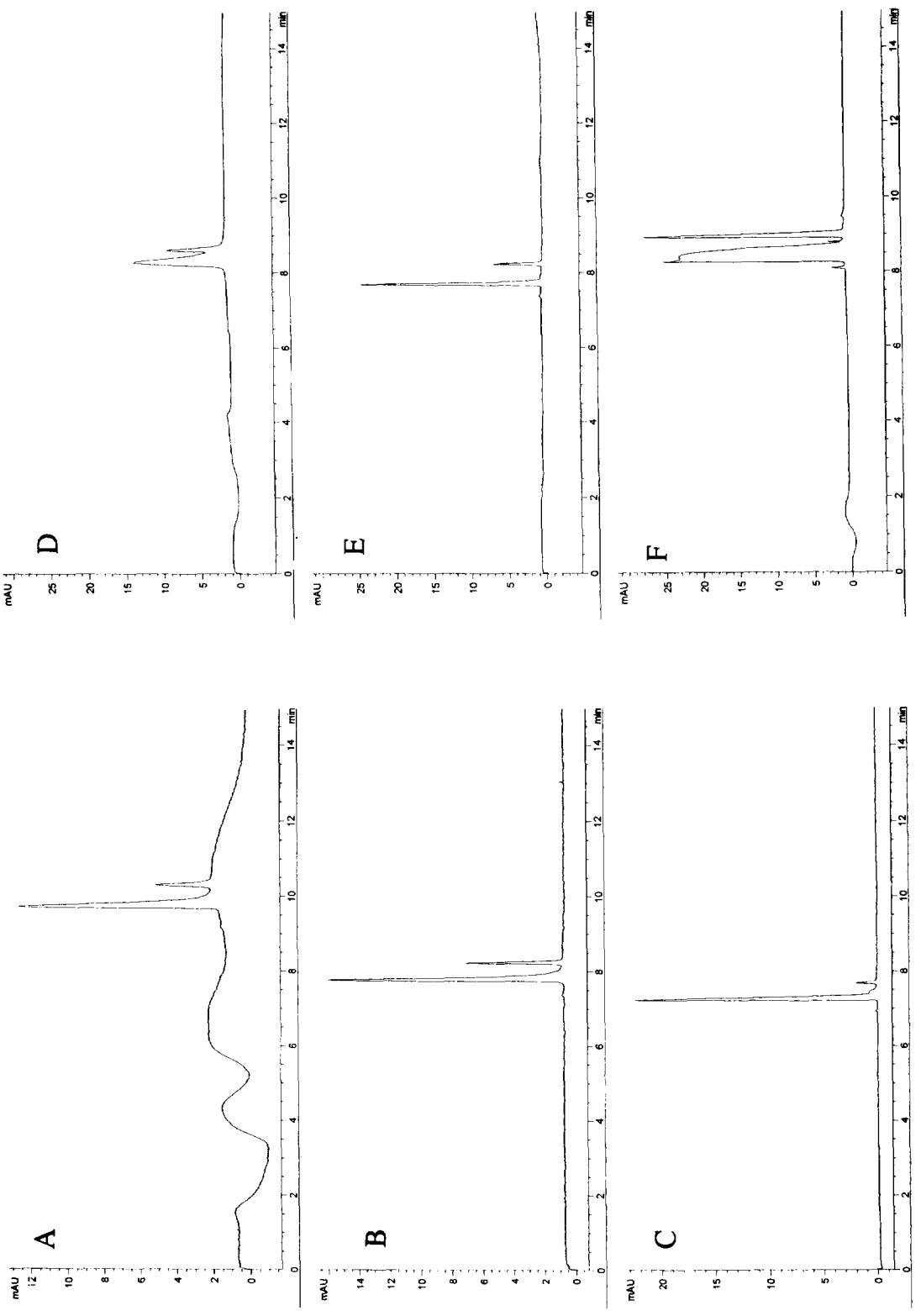


Fig. 2. CE of partly degraded tetracycline hydrochloride using different electrolytes. Electrophoresis medium: MeOH–MeCN (50:50, v/v) with different electrolytes added. (A) 27 m*M* methanesulphonic acid; (B) 25 m*M* ammonium acetate and 27 m*M* methanesulphonic acid; (C) 25 m*M* ammonium acetate, 10 m*M* citric acid and 27 m*M* methanesulphonic acid; (D) 25 m*M* ammonium formate and 27 m*M* methanesulphonic acid; (E) 25 m*M* lithium chloride and 27 m*M* methanesulphonic acid; (F) 25 m*M* ammonium acetate, 5 m*M* EDTA, 27 m*M* methanesulphonic acid. Injection for 5 s at 50 mbar. Capillary: 64 cm×50 μm I.D.; 55.5 cm to the detector. Temperature: 25°C. Voltage: 25 kV. Detection: 214 nm.

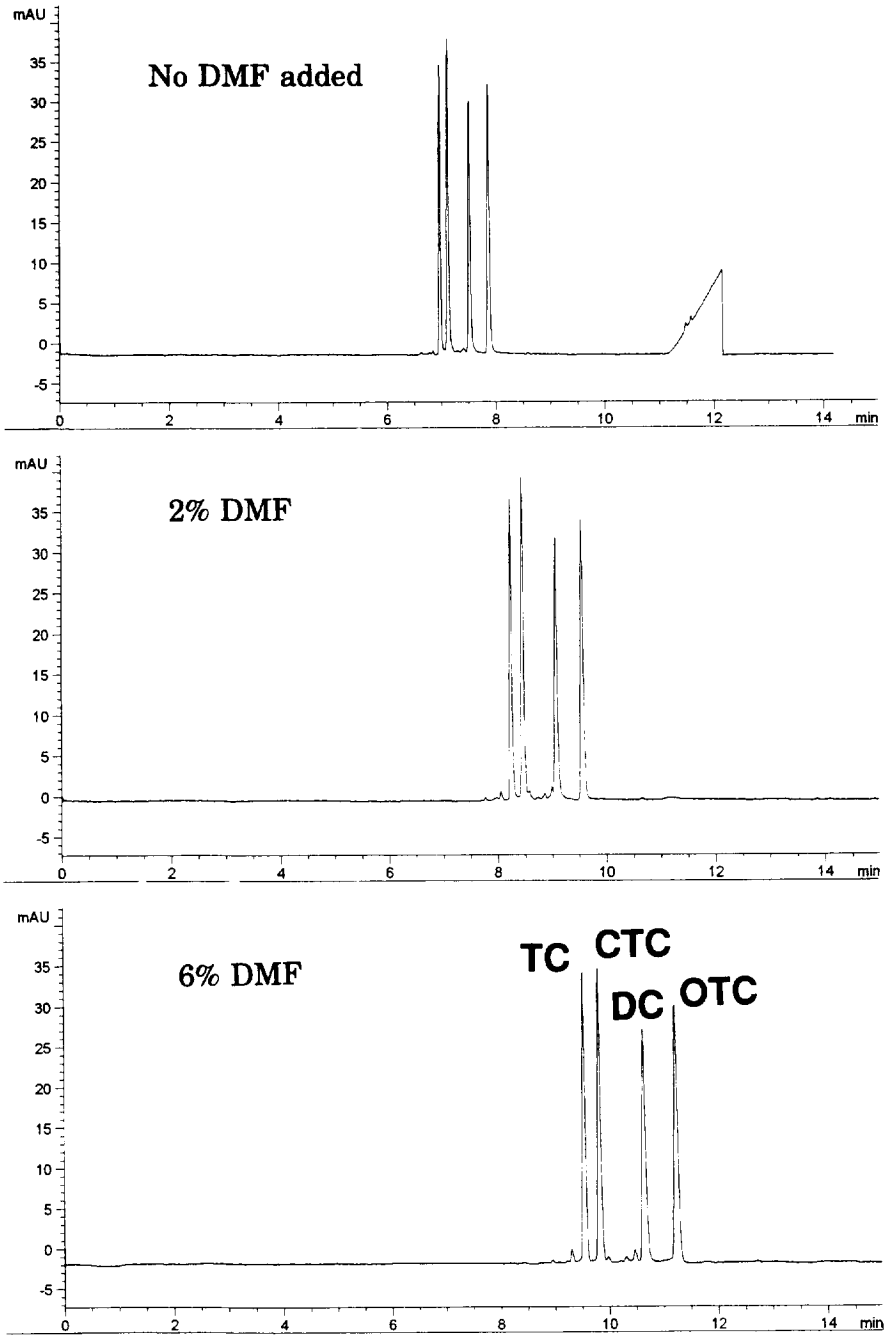


Fig. 3. Electropherograms of a mixture of some tetracycline antibiotics each in a concentration of 0.2 mg/ml using various mixtures of solvents. The samples were dissolved in MeOH. Injection for 3 s at 50 mbar. Capillary: 64 cm×50 μm I.D.; 55.5 cm to the detector. Electrophoresis medium was MeOH–MeCN (48:52, v/v) with the solvent as indicated on the electropherograms and 25 m*M* ammonium acetate, 10 m*M* citric acid and 118 m*M* methanesulphonic acid added. Temperature: 25°C. Voltage: 25 kV. Detection: 254 nm.

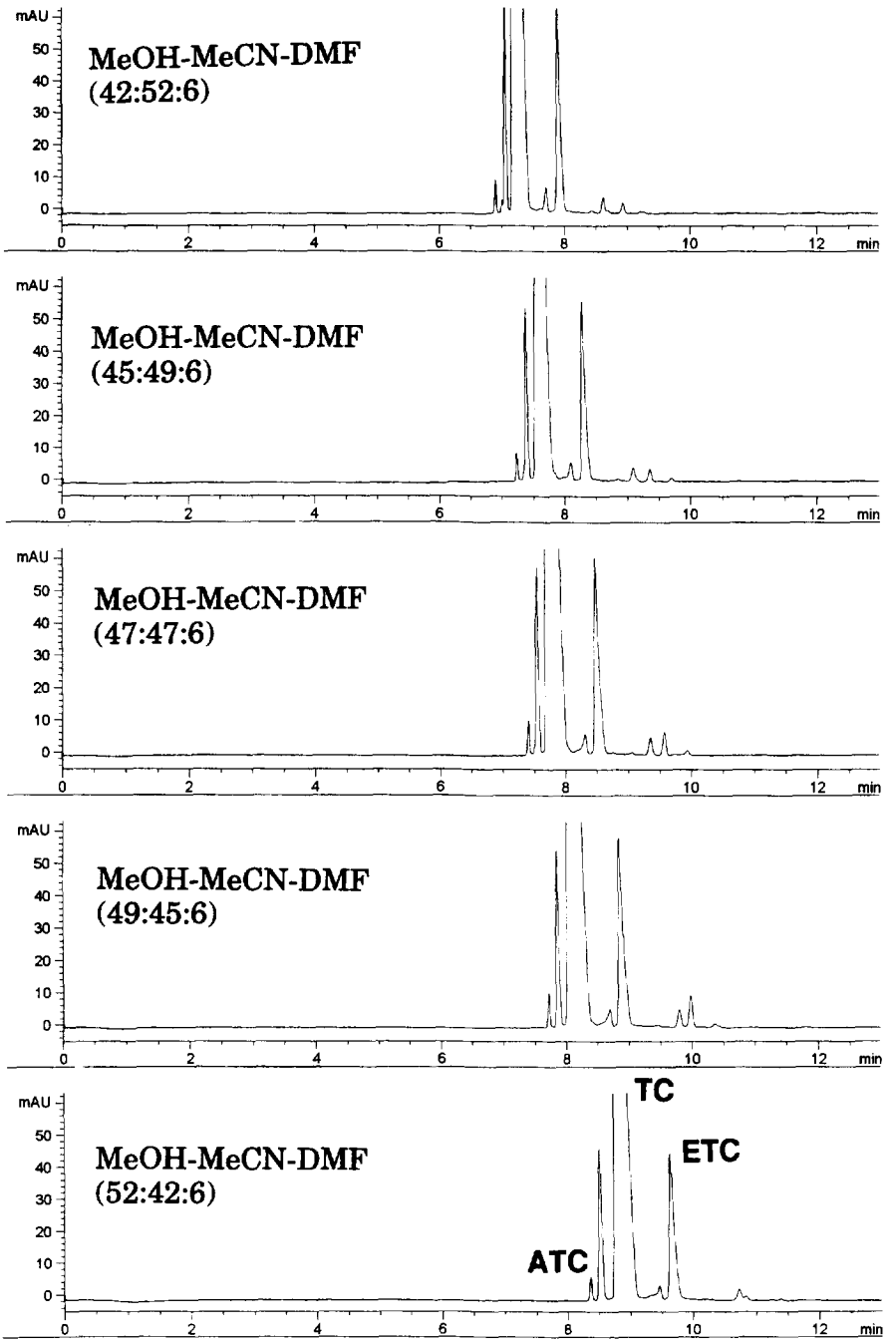


Fig. 4. Electropherograms of tetracycline hydrochloride 5.0 mg/ml using various mixtures of solvents. The samples were dissolved in MeOH–MeCN–DMF (47:47:6, v/v). Injection for 1 s at 50 mbar. Capillary: 64 cm×50 μm I.D.; 55.5 cm to the detector. Electrophoresis medium as indicated on the electropherograms with 25 m*M* ammonium acetate, 10 m*M* citric acid and 118 m*M* methanesulphonic acid added. Temperature: 25°C. Voltage: 30 kV. Detection: 254 nm.

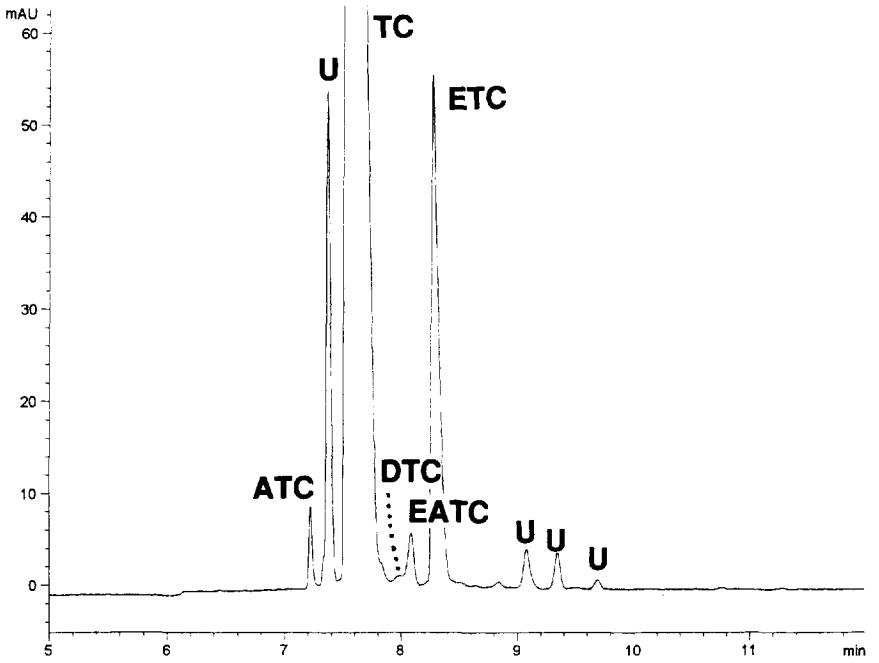


Fig. 5. Electropherogram of tetracycline hydrochloride raw material 5.0 mg/ml. The sample was dissolved in MeOH–MeCN–DMF (45:49:6, v/v). Injection for 1 s at 50 mbar. Capillary: 64 cm×50 μm I.D.; 55.5 cm to the detector. Electrophoresis medium: MeOH–MeCN–DMF (45:49:6, v/v) with 25 m*M* ammonium acetate, 10 m*M* citric acid and 118 m*M* methanesulphonic acid added. Temperature: 25°C. Voltage: 30 kV resulting in a current of approx. 65 μA. Detection: 254 nm. U=unknown.

aqueous CE are good and comparable with the data obtained using aqueous CE systems [12–14] but the repeatability is slightly poorer than in the HPLC methods using polymer-based column packing materials [9–11]. ATC as well as EATC can be quantitated at a concentration of 0.005 mg/ml and ETC at a concentration of 0.015 mg/ml with relative standard deviations of about 4%. Table 2 shows the amounts of impurities found in TC raw materials. It should be noted that the determination of DTC only

Table 1
Repeatability and linearity of the method

	TC	ATC	EATC	ETC
Repeatability of peak area (n=6) expressed as R.S.D. (%)				
Concentration level				
5.0 mg/ml	3.9	n.d.[a]	n.d.	n.d.
0.5 mg/ml	7.3	n.d	n.d	n.d
0.05 mg/ml	n.d	13.0	4.1	4.7
0.015 mg/ml	n.d.	3.9	4.9	4.0
0.005 mg/ml	n.d.	5.5	3.4	n.r.[b]
Linear regression				
Used range (mg/ml)	0.25–5.0	0.005–0.05	0.005–0.05	0.01–0.05
Number of observations (*n*)	10	5	5	5
Correlation coefficient of the regression analysis (*r*)	0.9980	0.9929	0.9970	0.9977

Conditions as in Fig. 5.
[a]n.d.=not determined.
[b]n.r.=no response obtained.

Table 2
Impurities found in TC from two different suppliers

Impurities	Supplier 1	Supplier 2
ATC	0.3%	0.7%
EATC	0.3%	1.0%
ETC	1.2%	7.6%
DTC	0.5%	0.3%

Conditions as in Fig. 5 with the exception of the injection time set to 3 s.

is based on one standard addition as only a limited amount of the compound was available. When the injection time was raised from 1 to 3 s a corresponding decrease in detection limits were obtained as only a minor band broadening due to the increased sample size was observed.

4.2. Suitability of the apparatus

As in aqueous CE, evaporation of solvent from the samples as well as from the electrophoresis medium must be given a serious thought regarding qualitative as well as quantitative measurements. The effects of evaporation may be more pronounced when using solvents more volatile than water. The robustness of the method was evaluated using three different CE systems. The electrophoresis medium consisted of MeOH–MeCN–NMF (47:47:6, v/v) with 25 m*M* ammonium acetate, 10 m*M* citric acid and 118 m*M* methanesulphonic acid added. The sample solute was dissolved in the solvent mixture used for the electrophoresis medium. The HP^{3D} system gave reproducible peak areas during 15 h when injecting from the same sample vial with 1-h intervals and using the same electrophoresis medium. The increase in the peak area of TC after 1 and 2 h was due to co-migration of TC and an unknown impurity. The migration times of TC, ETC, ATC and EATC were fairly repeatable but the elution order of an unknown impurity and TC was reversed probably due to an altered composition of the electrophoresis medium caused by evaporation of solvents. Evaporation of sample solvent as well as the electrophoresis medium makes it difficult to obtain repeatable quantitative data using the Beckman P/ACE 5010 system. The sample and the electrophoresis medium had evaporated to a level below the capillary ends after 4 and 6 h, respectively. Thus the peak areas at 4–6 h after first injection are zero as no sample was injected due to evaporation. The Waters Quanta 4000 system only allowed one run before the sample solution as well as the electrophoresis medium were evaporated to a level below the capillary inlet level. It should be noted that the capillary ends are positioned in the upper part of the vials in the Waters as well as in the Beckman CE system.

5. Conclusions

A highly selective method for separation of tetracycline and related compounds has been developed using non-aqueous CE. A good resolution of tetracycline and four of its well know impurities is obtained. Moreover, a number of unknown impurities can be detected. Four commercial available tetracycline antibiotics are completely separated. The nature of the organic solvents as well as the electrolytes have a major influence on the selectivity and the efficiency obtained in the electrophoretic system.

The evaporation of sample as well as electrophoresis medium have to be given serious thoughts regarding qualitative as well as quantitative measurements. Well closed sample and electrophoresis medium vessels have to be used in order to prevent evaporation of solvents. It was found that some CE systems are not well suited for quantitative measurements when using electrophoresis media with constituents more volatile than water due to evaporation of solvent from the sample as well as from the electrophoresis medium.

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