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Micellar electrokinetic capillary chromatography of macrolide antibiotics
Separation of tylosin, erythromycin and their related substances

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Abstract

The separation of tylosin by micellar electrokinetic capillary chromatography with a mixed micelle system is described. Good selectivity was obtained with sodium phosphate buffer (80 m*M*, pH 7.5) containing 20 m*M* sodium cholate and 7 m*M* cetyltrimethylammonium bromide (CTAB). This method permits tylosin to be separated from its closely related substances within 15 min. The influences of type of buffer, buffer pH, the concentrations of sodium cholate and CTAB were investigated. The robustness of the method was examined for tylosin by means of a full-fraction factorial design. Quantitative results are presented. Using a similar buffer system (80 m*M* sodium phosphate, pH 6.0, 20 m*M* sodium cholate and 5 m*M* CTAB), separation of erythromycin and its main related substances was also obtained. However, detection sensitivity and resolution are not sufficient for analysis of related substances in erythromycin commercial samples.

Keywords: Pharmaceutical analysis; Tylosins; Erythromycins; Antibiotics

1. Introduction

Tylosin and erythromycin are two members of the group of macrolide antibiotics, which are widely used against Gram-positive and some Gram-negative bacteria. Commercial samples of both antibiotics contain some closely related substances and several other minor components.

Analysis of macrolide antibiotics has been performed by paper chromatography, thin-layer chromatography as well as gas and liquid chromatography (LC). Among these methods, LC is the most popular technique for the determination of related substances and for assay. Recently, the perspectives of analysis of macrolide antibiotics were reviewed by Kanfer et al. [1]. Our laboratory has previously reported LC methods for the separation of tylosin and erythromycin from their related substances [2–4].

Capillary electrophoresis (CE) is a powerful separation and quantitation technique that often provides higher resolving power, shorter analysis time and lower operational cost than LC. In recent years, two

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papers were published using CE for the separation of different macrolide antibiotics [5,6]. Another paper published the separation of equal amounts of erythromycin A (EA) from five related substances with a buffer containing a very high concentration of salt and organic solvent in a long analysis time [7]. In real samples, the ratio of EA versus its related substances is much higher. This difference in ratio may affect the separation.

In micellar electrokinetic capillary chromatography (MEKC), different migration behavior and selectivity can be observed for various types of surfactants. For certain complex mixtures of structurally similar compounds, it can be difficult to find a suitable surfactant type that can provide enough selectivity and a sufficiently broad elution window. In these situations, the use of mixed micelles can lead to enhanced separations. Mixing surfactants with different interactive properties and selection of optimum composition can lead to great changes in selectivity for a given mixture. The size of the elution window for certain mixed micellar systems is often larger than that for individual surfactants.

The present paper reports the development and validation of a CE method using a mixed micellar system for tylosin. It enables the separation of tylosin and eight of its related substances within a much shorter time (including the washing procedure) than LC. The robustness of the system was examined by applying a full-fraction factorial design at two levels. Quantitative data are also reported.

Using a buffer system similar to that of tylosin, EA can be separated from its main related substances (when present in equal amounts). EA lacks a significant chromophore and has a low solubility in aqueous buffers, therefore satisfactory results for the determination of its related substances in commercial samples were not achieved. To improve resolution and sensitivity of detection, non-aqueous CE was examined, but again, no separation was obtained.

2. Experimental

2.1. Instrumental and operating conditions

CE experiments were carried out on a Spectraphoresis 1000 equipment (Thermo Separation Products, Fremont, CA, USA), which was driven by CE software (version 3.0.1) operating under IBM OS/2 TM (version 1.2). The vacuum system of the instrument applies a constant negative pressure of 5171 Pa for sample injection. Hydrodynamic injection was performed for 3 s during the method development and robustness study. UV detection was at 280 nm for tylosin and at 205 nm for erythromycin. pH measurements were performed on a Consort C831 pH-meter (Turnhout, Belgium). The pH of buffers was adjusted using phosphoric acid before making up to volume. All samples were dissolved in buffer [without sodium cholate and cetyltrimethylammonium bromide (CTAB)]. The capillary was washed at the beginning of the day with 0.1 *M* NaOH for 5 min followed by a water wash for 5 min at 60°C. Before every analysis, the capillary was washed for 2 min with running buffer.

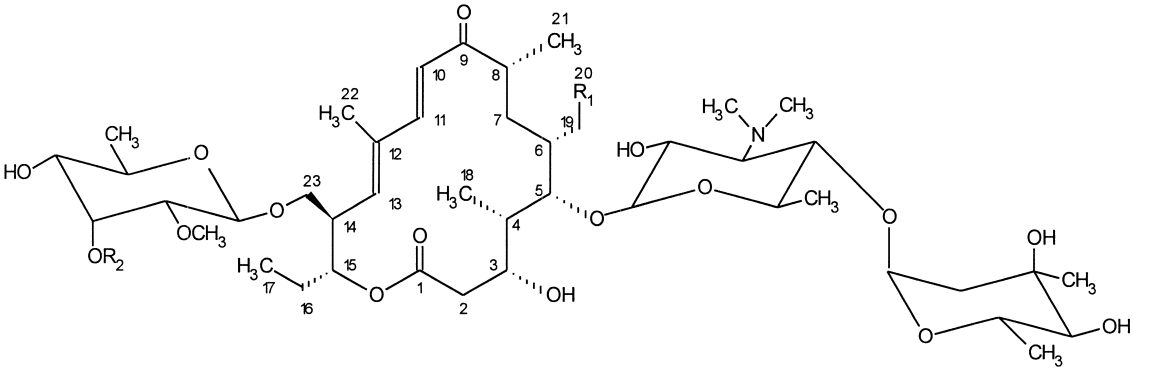
2.2. Materials and related substances

Sodium cholate 99% (SC) was purchased from Acros Organics (Geel, Belgium), and CTAB was obtained from Merck (Darmstadt, Germany). All other reagents were also obtained from Merck or Acros Organics. The fused-silica capillary was from Polymicro Technologies (Phoenix, AZ, USA): 44 cm (effective length 36 cm)×50 μm I.D. Throughout the study, Milli-Q water was used (Millipore, Milford, MA, USA). All the solutions were filtered through 0.2-μm nylon filters (Euroscientific, Lint, Belgium).

A laboratory standard for tylosin A (TA) was available in the laboratory. The purity of this standard is 90.3% (w/w). Small amounts of desmycocin or tylosin B (TB), macrocin or tylosin C (TC), relomycin or tylosin D (TD), demycinosyltylosin (DMT), 5-*O*-mycaminosyltylonolide (OMT), lactenocin (LACT) and 20-dihydrotylosin B (DHTB) were available as reference substances. Tylosin A aldol (TAD) and isotylosin A (isoTA) were isolated and purified as described by Paesen et al. [3]. All these structures are shown in Fig. 1. The reference substances of erythromycin were described before [4].

2.3. Software

The set-up of the applied full-factorial design,



	R_1	R_2	Mycarose	Mycinose
Tylosin A	CHO	CH_3	+	+
Tylosin B	CHO	CH_3	-	+
Tylosin C	CHO	H	+	+
Tylosin D	CH_2OH	CH_3	+	+
Lactenocin	CHO	H	-	+
OMT	CHO		-	-
DMT	CHO		+	-
DHTB	CH_2OH	CH_3	-	+

+ = sugar present; - = sugar not present

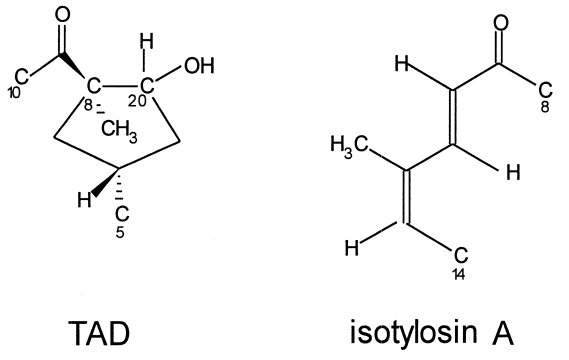


Fig. 1. Structures of tylosin and its related substances.

together with the analysis of the measured response variables and the multivariate regression calculation, was supported by the statistical graphics software system Statgraphics version 6.0 (Manugistics, Rockville, MD, USA).

3. Results and discussion

3.1. Separation of tylosin

The impurities most frequently detected in commercial samples of tylosin are TB, TC, TD and DMT. Other related substances have been isolated, e.g., OMT, LACT and DHTB. Other minor impurities, which may occur in solutions of tylosin, are TAD and isoTA. Method development was performed with a commercial sample containing all the compounds mentioned above. In preliminary experiments, solutions of different surfactants such as sodium dodecyl sulfate (SDS), Triton X-100, CTAB and SC in phosphate buffer were tried out as background electrolyte. It appeared that none of them gave sufficient selectivity. So, the use of mixed micelles was attempted. When CTAB and SC were introduced together, a clear improvement of separation was observed. Because selectivity greatly depends on the type of buffer, three different buffers were also compared, namely sodium carbonate, sodium tetraborate and sodium phosphate. Each was prepared at a concentration of 80 m*M* and also contained 20 m*M* SC and 10 m*M* CTAB. The pH of all solutions was 6. Sodium carbonate caused a broadening of the peaks. Using tetraborate, the baseline and resolution were poorer. Subsequent experiments were thus performed with sodium phosphate because it gave the best separation. The influences of buffer pH (6–8, steps 0.5), the concentration of CTAB (3–12 m*M*, steps 1 m*M*) and SC (16–23 m*M*, steps 1 m*M*) were investigated. The four most important compounds, TA, TB, TC and TD, presented similar separation at pH 6.5, 7 and 7.5. But at pH 7.5, more small impurities could be separated. So this pH was chosen. It was found that the concentration of CTAB and SC are parameters with a more substantial influence on migration time than pH. Adjustment of the ratio of SC/CTAB affects selectivity significantly. Their concentration effects were checked very carefully. In this work, an increase in the concentration of SC decreases the migration time of all compounds, which is opposite to the effect noticed in a buffer without CTAB [5]. A possible explanation is that, due to the formation of mixed micelles of SC and CTAB, the concentration of free CTAB decreases with an increase in the SC concentration. This would then result in an electroosmotic flow (EOF) increase, which was noticed indeed in our experiments, and this effect must be dominant over interaction between SC and the compounds. This also provides an explanation for the effect of CTAB in this buffer system. An increase in the concentration of CTAB increases migration time and there is a threshold concentration value, above which the EOF is reversed, as usual. However, this limit value is much higher when SC is used in the buffer in contrast to a buffer without SC (about 0.3 m*M* [8]). In this work, CTAB provided a wider elution window. This is probably due to a reduction of EOF by CTAB which caused the mixed micelles to migrate slowly. The EOF was not reversed so that the normal voltage polarity could be kept.

The effects of buffer concentration, of organic modifiers such as acetonitrile and methanol and of α-, methyl-β- and γ-cyclodextrin were investigated. None of them provided improved separation. Instrumental parameters such as capillary temperature and applied voltage were optimized. Finally chosen electrophoresis conditions were as follows: running buffer, 80 m*M* Na_2HPO_4, 20 m*M* SC and 7 m*M* CTAB adjusted to pH 7.5; voltage, 15 kV; temperature, 25°C. The method permits one to separate TA from eight of its impurities and all these from each other within 10 min. Unfortunately, the minor impurity isoTA co-migrated with TA under all conditions. Fig. 2 shows a typical electropherogram of a tylosin commercial sample. The analytes migrate faster than t_0.

3.2. Method robustness

Robustness is an important aspect of method validation. One evaluates the influence of small changes in the operating or environmental conditions of the analytical procedure on measured or calculated responses. The changes introduced when performing

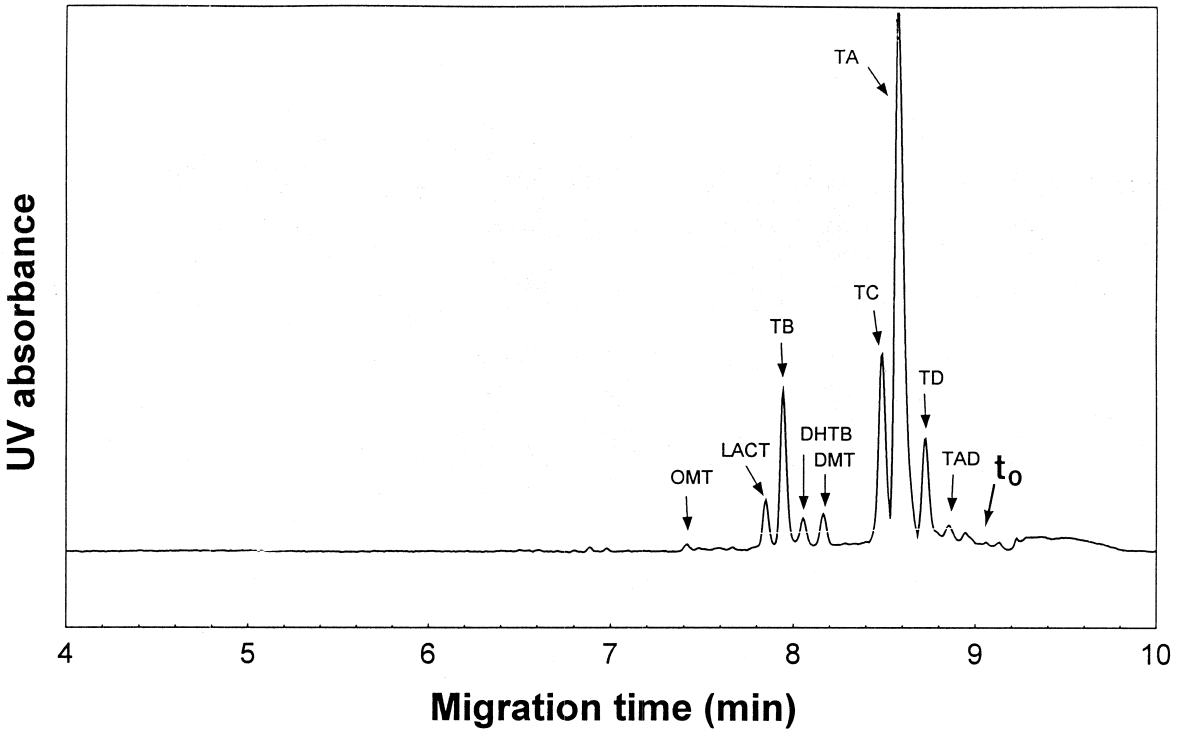


Fig. 2. Electropherogram of a commercial sample of tylosin (1 mg/ml). Running buffer, 80 m*M* sodium phosphate, 20 m*M* SC and 7 m*M* CTAB at pH 7.5; capillary: uncoated fused-silica, 44 cm (effective length 36 cm)×50 μm I.D.; temperature, 25°C; voltage, 15 kV; detection wavelength, 280 nm; hydrodynamic injection: 3 s.

a robustness test reflect the changes that can occur when a method is used in different laboratories, by different experimenters, or using different equipment [9].

Experimental designs involve simultaneous alteration of all parameters according to a predefined matrix of experiments. Among them, full-factorial designs are well adapted to the determination of the relative importance of each variable in comparison to the estimated responses. As a matter of fact, l^n experiments are needed to evaluate the influence of n variables by a full-factorial design at l levels.

In this study, the influence of each of three relevant electrophoretic parameters, governing the separation process, was examined by applying a full-fraction factorial design at two levels [10]. This involves $2^3=8$ different experimental measurements. One central point combination was included in the design and so nine measurements had to be performed as well as duplicate experiments 10–18. Randomization of runs was performed by the software system Statgraphics. One injection was done for each experiment and experiments were duplicated using the same buffer solution. In this work, response variables were analyzed ignoring interactions greater than two. The system would thus only estimate interactions between two parameters. The standard error was estimated using all degrees of freedom that remain after the system has estimated the desired effects.

The three relevant electrophoretic parameters examined as variables were: (1) concentration of CTAB; (2) concentration of SC; (3) buffer pH. The values for the design are given in Table 1. As response variables in the factorial design, selectivities among four most important compounds were measured, namely TA, TB, TC and TD (data not shown for TB). Analysis of the measured response variables enabled to obtain standardized pareto charts [10].

Table 1
Values corresponding to −1, 0 and +1 levels

Electrophoretic parameter	Low value (−1)	Central value (0)	High value (+1)
Concentration of CTAB (m*M*)	5	7	9
Concentration of SC (m*M*)	18	20	22
Buffer pH	7.0	7.5	8

A standardized pareto chart consists of bars with a length proportional to the absolute value of the estimated effects, divided by the standard error. The codes A, B and C correspond to the concentration of CTAB, the concentration of SC and the buffer pH respectively. The combination of two codes indicates the interaction effect between the two parameters. The bars are displayed in order of the size of the effects, with the largest effects on top. The chart includes a vertical line at the critical *t*-value for an α of 0.05. Effects for which the bars are smaller than the critical *t*-value are considered as not significant and do not affect the response variables. Effects may be positive or negative.

Fig. 3 shows influences of the parameters on the selectivities (a) between TC and TA (α_{TC-TA}) and (b) between TA and TD (α_{TA-TD}). The related substances TC and TD were chosen because they migrate most closely to the main component TA. From Fig. 3a, it can be seen that only the buffer pH has a negative influence on α_{TC-TA}. This means that a decrease in pH improves the selectivity α_{TC-TA}. None of the other parameters and interactions between parameters are significant. Fig. 3b shows that the concentration of CTAB has a negative influence on α_{TA-TD}. Although the buffer pH has no significant influence, there is a significant negative interaction with the concentration of CTAB. This means that a change in pH affects α_{TA-TD} more at a lower concentration of CTAB.

From the migration times recorded during the robustness study, it can also be concluded that the method is robust, because within the range checked, the separation among all four important compounds can be maintained.

3.3. Quantitative analysis of tylosin

The quantitative features of this method were examined and the results are shown in Table 2. Repeatability studies were performed with a 1 mg/ml solution being injected for 3 s, which is suitable for assay of tylosin. In limit of detection (LOD) and limit of quantitation (LOQ) tests, a solution of tylosin (2.0 mg/ml) was diluted gradually. The

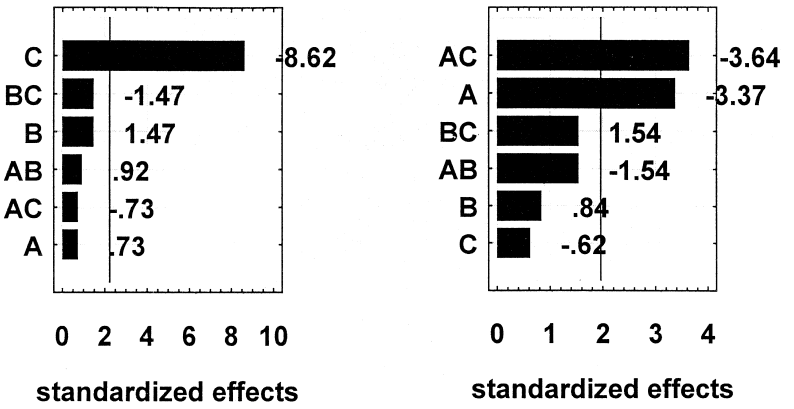


Fig. 3. Standardized pareto charts, representing the estimated effects of parameters and parameter interactions on the selectivities (a) between compounds TC and TA, (b) between compounds TA and TD.

Table 2
Quantitative features for MEKC of tylosin

Parameter	Tylosin
Within-day repeatability ($n=6$)[a]	
Migration time	RSD=0.2%
Corrected area	RSD=1.7%
Day-to-day repeatability ($n=18$)[a]	
Migration time	RSD=1.6%
Corrected area	RSD=2.0%
Linearity: y=corrected area, x=tylosin concentration (mg/ml), range=0.25–1.50 mg/ml, number of concentrations=6, total number of analyses=12	$y=32\ 765x+548$, $r=0.998$, $S_{y,x}=981$
LOD (S/N=3)[b]	8.5 pg (0.025%, relative)
LOQ, RSD=9.7% ($n=6$)[b]	17 pg (0.05%, relative)

[a] 1 mg/ml, hydrodynamic injection, 3 s.
[b] 2 mg/ml, hydrodynamic injection, 10 s.

solutions with a dilution of 0.025% relative to the originally injected solution, and 0.05% relative to the originally injected solution, correspond to the LOD and LOQ, respectively. The injection volume was about 17 nl for LOD and LOQ tests.

3.4. Separation of erythromycin

In commercial samples of erythromycin, the most important impurities are erythromycin B (EB), erythromycin C (EC), erythromycin E (EE) and pseudoerythromycin A enol ether (psEAEN). During method development for erythromycin, a similar mixed micelle system was used as for tylosin. The influence of buffer pH, concentration of CTAB and SC were investigated using the same approach. As a result of this development work, the following electrophoretic conditions were chosen: running buffer, 80 m*M* sodium phosphate including 20 m*M* SC and 5 m*M* CTAB adjusted to pH 6.0; voltage, 18 kV, temperature, 25°C, UV detection at 205 nm. Under these conditions, EA can be separated from its important related substances EB, EC, EE and psEAEN used at equal concentrations (electropherogram not shown). The order of migration is EC, EE, EA, psEAEN and EB. Erythromycin lacks a significant chromophore and has a low solubility in aqueous buffer; insufficient amounts can be applied for the determination of its related substances in commercial samples.

Non-aqueous CE (NACE) was also tried for erythromycin. An important aspect is the high solubility of erythromycin in some organic solvents, which can then be more easily detected. However, EA, EB, EC and EE always co-migrated in methanol–acetonitrile–ammonium acetate or sodium acetate buffer systems. A possible explanation is that the ionization of the compounds was suppressed in these two organic solvents. Because of the very low detection wavelength needed for erythromycin, other types of organic solvents for NACE could not be used.

4. Conclusion

A MEKC method using a mixed micellar system was developed for separation of tylosin from its related substances. A comparison with LC [2] shows that the MEKC method yields quantitative results similar to LC and in much less time than LC. The selectivity is worse in CE for separation of TC and TA. Nevertheless, there is great potential in the use of CE as an alternative tool for analysis of tylosin. For another macrolide antibiotic, erythromycin, insufficient sensitivity can be obtained for the determination of the related substances.

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