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Sample stacking for the analysis of penicillins by microemulsion electrokinetic capillary chromatography

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Abstract

We present a method for determining eight penicillin antibiotics using microemulsion electrokinetic chromatography (MEEKC). We studied how the composition of the microemulsion affected separation by modifying such parameters as the surfactant or the addition of organic solvents. The best microemulsion system consisted of 0.5% ethyl acetate, 1.2% 1-butanol, 2% Brij 35, 10% 2-butanol and 86.3% 10 mM borate buffer at pH 10. We studied the suitability of this microemulsion composition for analyzing a commercial drug. To improve the sensitivity of the method, we used the stacking technique reversed electrode polarity stacking mode (REPSM), which increased the detection limits by about 40-fold.

Keywords: Antibiotics; Capillary electrophoresis; Penicillins; Microemulsion electrokinetic chromatography; Preconcentration

1. Introduction

An antibiotic is any chemical compound that is used to kill or inhibit the growth of infectious organisms, particularly bacteria and fungi. It is generally believed that there is a link between the use of antibiotics in animal fodder, bacterial resistance to these drugs and human diseases [1]. In particular, several penicillin-group antibiotics with various chemical structures are widely used to treat infectious diseases in humans and animals [2]. The presence of these compounds in food chains can lead to the development of allergic reactions and new strains of bacteria that are resistant to antibiotics. These risks led to the legislation of these antibiotics by the Council Regulation of the European Community 2377/90/EC [3]. Annex I of this regulation (updated 22/12/04) establishes the maximum limits of eight penicillins in animal tissues.

Most analytical methods for determining penicillin compounds are based on liquid chromatography [4–10]. Recently, several papers have described how capillary electrophoresis (CE) [1,4,11–19] can be used to analyze these compounds, mostly with micellar electrokinetic chromatography (MEKC) [12–15,17–20] because of its good selectivity and wide applicability.

In the last few years, a technique known as microemulsion electrokinetic chromatography (MEEKC) has been used to insert microemulsions as alternative pseudo-stationary phases in electrokinetic separations [21]. As with MEKC, the technique separates solutes on the basis of their hydrophobicities and electrophoretic mobilities [22] but with different selectivity [21]. Microemulsions are solutions containing dispersed nanometer-sized droplets of an immiscible liquid [23]. Typically, droplets consist of an immiscible oil suspended in water. There is a high surface tension between the layers of immiscible liquids that prevents them from mixing. A surfactant, which is soluble in both layers because it contains both hydrophilic and hydrophobic portions, is added to coat the oil droplets formed. The oil drops are coated in order to reduce the surface tension between the two liquids. The surface tension is further lowered by adding a short-chain alcohol called a co-surfactant, which stabilizes the microemulsion system [22,23]. Therefore, a typical microemulsion used for MEEKC may consist of 0.8% *n*-octane, 3.3% sodium dodecyl sulfate (SDS), 6.6% 1-butanol and 89.3% 10 mM borate buffer at pH 9.2 [24–27]. It should be taken into account that solutes in MEEKC are more able to penetrate the surface of the droplet than the surface of a micelle, which is much more rigid. This means that MEEKC can be applied to a wider

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range of solutes, including neutral and charged compounds, than MEKC.

One of the advantages of MEEKC is that it takes into account many parameters: the type and concentration of the oil, buffer, surfactant, co-surfactant, counter-ion and the pH all affect the separation performance [26]. As well as the mostly aqueous electrolyte solution and the surfactant responsible for stabilizing the oil droplets, various organic solvents with different properties play an important role in the composition of the microemulsions used in MEEKC [28].

Although MEEKC has been used to separate some penicillins [29], to the best of our knowledge, it has not yet been used to separate the analytes studied in this paper. Altria et al. [29] showed that MEEKC could be used to analyze penicillin compounds. They separated penicillin G and penicillin V from a mixture of several cephalosporins in an analysis time of less than 4 min.

To verify if MEEKC has any advantages over MEKC in terms of resolution and analysis time, this paper studies the potential of MEEKC to separate and determine the eight legislated penicillins used as veterinary drugs. The microemulsion parameters (pH, the nature of the surfactant, the temperature of the capillary, the nature and concentration of the buffer and the addition of organic solvents) were optimized. We evaluated the usefulness of the method by analyzing a commercial drug sample.

Because CE capillaries are small, only very small sample volumes can be loaded into the column. CE is therefore not a very sensitive technique. To preconcentrate samples and increase the amount of sample that can be loaded into the column without degrading the separation, several techniques have been developed in the various electrophoretic modes [19,30,31]. One of them is the reversed electrode polarity stacking mode (REPSM), which has been used as a stacking technique in MEKC [19,31]. REPSM introduces the sample into the capillary hydrodynamically. A stacking voltage is then applied at negative polarity to preconcentrate analytes at the interface between the sample zone and the background electrolyte, and the sample matrix is pumped out from the capillary by EOF. This technique was first coupled to MEEKC to analyze some NSAIDs by Macià et al. [32]. In the present paper, we study how the on-line coupling of REPSM-MEEKC can be used to analyze penicillins at trace levels for the first time.

2. Experimental

2.1. Chemicals

Penicillin V potassium salt was purchased from Riedel-de-Häen (Seelze, Germany). Sodium dodecyl sulfate (SDS), amoxicillin, dicloxacillin sodium salt, nafcillin and sodium cholate were obtained from Sigma (Saint Louis, USA). Penicillin G potassium salt, oxacillin sodium salt, cloxacillin sodium salt, ampicillin sodium salt, sodium tetraborate and polyethylene glycol dodecyl ether (Brij 35) were obtained from Fluka (Buch, Switzerland). Sodium hydroxide and hydrochloride acid 35% were obtained from Prolabo (Bois, France). Ethyl acetate and 1-butanol were obtained from Merck (Darmstadt, Germany), octane and methanol from SDS (Peypin, France), Tris(hydroxymethyl) aminomethane (Tris) buffer and dibutyl tartrate from Aldrich (Steinheim, Germany) and *n*-heptane from Probus (Badalona, Spain). Water was obtained from a Millipore Milli-Q system (Millipore, Bedford, USA).

2.2. Equipment

MEEKC analyses were performed using a Hewlett Packard ^{3D}CE Capillary Electrophoresis System (Hewlett-Packard, Palo Alto, CA, USA) equipped with an on-column diode-array detector, an autosampler and a power supply able to deliver up to 30 kV. A HP ChemStation (Agilent) version A:04.01 was used for instrument control, data acquisition and data analyses.

2.3. MEEKC conditions

Separations were performed on 45-cm long (detection window at 36.5 cm), 75-μm internal diameter, uncoated fused-silica capillaries (Supelco, Bellefonte, USA). Unless otherwise specified, the capillary was thermostated at 25 °C, the voltage was kept constant at 10 kV during analysis and the detection wavelength was 210 nm. Normal sample injection was carried out using the pressure mode for 5 s at 50 mbar.

New capillaries were conditioned with 1 M sodium hydroxide for 5 min at 60 °C, water for 10 min and electrolyte for 30 min at 25 °C. At the beginning of each day, the capillaries were rinsed successively with 0.1 M sodium hydroxide, then with water for 8 min each and finally with microemulsion solution for 10 min. The capillaries were rinsed between injections with the microemulsion solution for 2 min. When not in use, the capillaries were washed with 0.1 M sodium hydroxide, with water for 8 min, and then dry stored.

2.4. Buffers and standards

Microemulsions were prepared by weighing the appropriate ratio of components to obtain different compositions. The order of addition was the same in all cases: initially the oil was mixed with the co-surfactant, and then the surfactant and the buffer were added. When organic solvents were used, they were added before the surfactant. The mixture was sonicated for 30 min to aid complete dissolution and an optically transparent microemulsion was formed. The pH was adjusted using 1 M NaOH or HCl 35%. Before use, the microemulsion solutions were filtered through a 0.22 μm microfilter. The solution remained transparent and stable for several weeks.

Stock standard solutions of penicillins were prepared by dissolving each compound in Milli-Q water to obtain a concentration of 1000 mg L^{-1}. For MEEKC experiments, a standard mixture of 100 mg L^{-1} was prepared and working solutions were made by dissolving a volume of this solution in water to obtain the final concentrations.

A commercial pharmaceutical preparation containing amoxicillin was used to test the suitability of the method for industrial samples. The individual solution was prepared by dissolving a quantity of the preparation in water to give an amoxicillin concentration of 100 mg L^{-1}. This was then diluted to obtain a final concentration of 10 mg L^{-1}.

All the solutions were filtered through a 0.22 μm membrane filter prior to use.

2.5. REPSM procedure

After the capillary had been conditioned with the microemulsion separation solution, hydrodynamic injection was performed at 50 mbar for 270 s. The injection end of the capillary was then placed on a microemulsion separation solution and a voltage of −5 kV was applied. When the current was 95% of that reported when the capillary was filled only with background solution, the voltage was turned off and the separation began.

3. Results and discussion

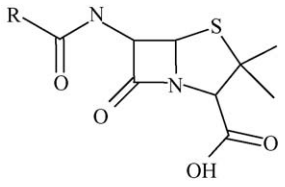
In a previous study, we developed a simultaneous MEKC separation of the eight legislated penicillins [19]. Now, in this study, one of our main aims was to evaluate the potential of MEEKC for separating these penicillins and compare the results with those obtained by MEKC.

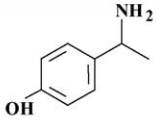
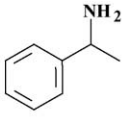
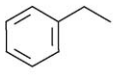
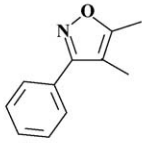
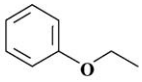
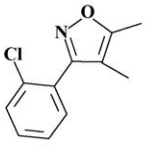
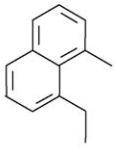
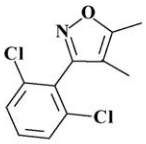
It has been shown that a microemulsion consisting of 0.8% octane (oil), 6.6% 1-butanol (co-surfactant), 3.3% SDS (surfactant) and 89.3% tetraborate (buffer) can be used at pH 9.31 for a wide range of drugs [29]. We therefore selected this mixture to explore the potential of MEEKC at separating penicillins. To study the separation, we investigated the effect of the pH, the temperature of the capillary, the concentration of the co-surfactant, the type of surfactant, the type and concentration of the buffer and the addition of organic solvents, taking the system mentioned as the starting point. The voltage applied was 10 kV.

3.1. Effect of microemulsion pH

The pH of the microemulsion has a strong effect on separation selectivity because it affects both solute ionization and the level of EOF generated [33]. Table 1 shows the structure of

Table 1
Structure and pK_a of the studied compounds[a] [34]



Compound	R	pK_{a1}	pK_{a2}	pK_{a3}
Amoxicillin		2.61	7.2	9.6
Ampicillin		2.62	7.4	–
Penicillin G		2.62	–	–
Oxacillin		2.61	–	–
Penicillin V		2.62	–	–
Cloxacillin		2.60	–	–
Nafcillin		2.61	–	–
Dicloxacillin		2.60	–	–

[a] Calculated using Advanced Chemistry Development (ACD/Labs) Software Solaris V8.14 (© 1994-2005 ACD/Labs).

the compounds investigated with the corresponding pK_a. Acidic drugs like penicillins typically have pK_a values in the region of pH 2.5–6, so they are ionized at a pH above 6. In order to ensure that the compounds were negatively charged and to maximize the EOF, we selected pH 10. Acidic solutes have a negative electrophoretic mobility and partition into the droplet, but they are repelled from it because solutes and micelles are both negatively charged [33].

3.2. Effect of co-surfactant concentration

The co-surfactant is normally added to decrease the surface tension between the nanodroplet and the aqueous phase. This additive affects the partition coefficients of the analytes and has an impact on migration and separation selectivity. The migration times can be altered by varying the co-surfactant concentration; this affects the solution viscosity, which in turn affects the EOF. The size of the oil droplet increases as the co-surfactant concentration increases, which reduces the charge density of the droplet and makes it less able to oppose the EOF [22,23]. 1-Butanol is the most frequently used co-surfactant in MEEKC [23]. To investigate the effect of co-surfactant concentration on the separation of penicillins, we tested various concentrations between 5.6 and 7.6% (6.6% is the most common concentration of 1-butanol and, at around this value, the microemulsion is stable). We observed that the method selectivity changed as the co-surfactant concentration changed so, although the analysis times were similar, the separation between the peaks was different. Results were best with 6.6% of 1-butanol.

3.3. Effect of capillary temperature

We tested several temperatures between 20 and 35 °C and no change in the migration order was observed. We kept 25 °C as the analysis temperature because there was less overlapping and migration times were not too long.

3.4. Effect of the nature of the surfactant

The nature of the surfactant has a marked effect on the separation; it influences the droplet charge and size, the level and direction of the EOF and the level of ion-pairing with charged solutes [22,23,33]. SDS is the most commonly used surfactant and 3.3% is the most common SDS concentration. As can be seen in Fig. 1A, with 3.3% SDS, the eight peaks were separated in less than 24 min.

To investigate how the nature of the surfactant affects migration times and selectivity, we tested another anionic surfactant, sodium cholate. Sodium cholate is an anionic bile salt surfactant that has also been used to generate negatively charged droplets. Fig. 1B shows that the resolution was worse for cholate than for SDS, since only seven peaks were separated and amoxicillin and cloxacillin co-eluted. The migration times were shorter than with SDS since the analysis time was 9 min lower. The migration order was almost the same, except for amoxicillin which, with SDS, eluted first and, with cholate, overlapped with cloxacillin in fifth place. This may be due to the structure of cholate. Some studies have suggested that cholate forms helical micelles, which leads to more polar aggregates than those formed with *n*-alkyl surfactants [35]. Amoxicillin was double charged, so its high polarity allowed it to remain longer inside the droplet.

Although the zero electrophoretic mobilities of neutral surfactants cannot be exploited in the MEEKC separation of non-ionic compounds, they can be used successfully in the separation of ionic compounds. The problems with Joule heating at increasing concentrations of ionic surfactants can be avoided by using non-ionic surfactants, which can be added to the buffer at higher concentrations. Voltages can also be high.

We also investigated the non-ionic surfactant Brij 35 [36]. One of the consequences of the neutrality of Brij 35 is that the droplet formed has no electrophoretic mobility of its own and migrates with the EOF. Fig. 1C shows the separation when this non-ionic surfactant is used. In comparison with cholate, we found that the resolution improved and eight peaks were obtained, although cloxacillin and nafcillin overlapped. The migration times were also much lower than those obtained with SDS, since the latest peak migrated at approximately 10 min. As we can see in Fig. 1C, the elution order was very different from that of the anionic surfactants. This may be because Brij 35 is a non-ionic surfactant, so there was no repulsion between the ionic charges of the solutes and the negative charges of the surface of the droplet, as there was with SDS.

Finally, we tested a mixture of SDS-Brij 35 [23,27,37]. Changing the surfactant of the microemulsion from SDS (Fig. 1A) to the mixture (1.65% SDS: 1.65% Brij 35; Fig. 1D) considerably reduced the separation window and shortened the analysis time (from 24 to 12 min), but seven of the eight

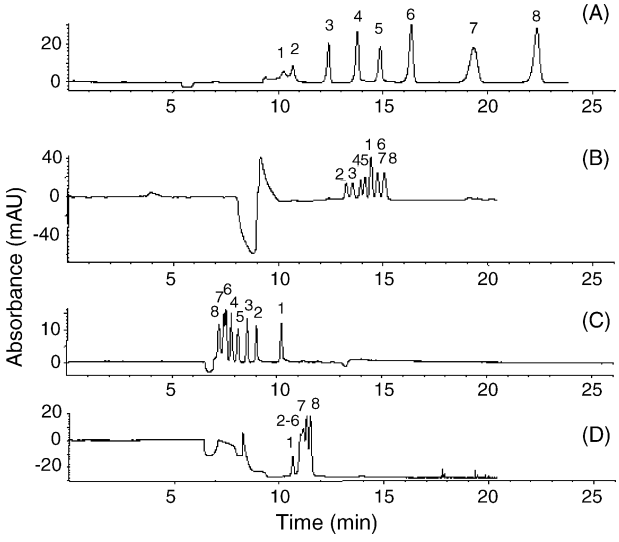


Fig. 1. Electropherograms obtained from a standard mixture of penicillins with different microemulsion systems. Separation conditions: 0.8% *n*-octane, 6.6% 1-butanol, 89.3% 10 mM borate buffer and (A) 3.3% SDS; (B) 3.3% sodium cholate; (C) 3.3% Brij 35; (D) 1.65% SDS-1.65% Brij 35 as surfactant; fused-silica capillary (36.5 cm effective length) thermostatted at 25 °C; hydrodynamic injection (50 mbar, 5 s); UV detection at 210 nm; voltage 10 kV. The analytes in a concentration of 10 mg L^{-1} are listed in order of increasing migration time: (1) amoxicillin, (2) ampicillin, (3) penicillin G, (4) oxacillin, (5) penicillin V, (6) cloxacillin, (7) nafcillin and (8) dicloxacillin.

compounds co-eluted. This was probably because the droplets formed in this case contained both a non-ionic and an ionic surfactant and the solute partition in the droplet was affected by the partition behavior with the two surfactants individually.

Results were best when SDS or Brij 35 were used individually. So further analyses were carried out with these surfactants.

3.5. Effect of oil type

n-Octane and *n*-heptane are the most commonly used oils in MEEKC. However, they lead to microemulsions with a high surface tension, which means that if the microemulsion is to be stabilized, the level of the surfactant must be high. When the microemulsion consists of *n*-octane (or *n*-heptane), 1-butanol, SDS and borate, separations are not fast because the concentration of SDS required to form droplets is high. When the ratio of surfactant increases, the EOF decreases which slows down the migration of the droplets and compounds to the detector.

Oils with a lower surface tension such as ethyl acetate and dibutyl tartrate have been used in MEEKC [25] so the microemulsion can be produced with less surfactant, which reduces the analysis time. In this way, we studied the effect of changing the inner organic phase in the microemulsion droplets from *n*-octane (or *n*-heptane) to ethyl acetate or dibutyl tartrate with two types of surfactants, SDS and Brij 35. The microemulsion solutions studied consisted of 0.5% lower surface tension oil, 1.2% 1-butanol, 0.6% SDS (or 0.6% Brij 35) and 97.7% 10 mM borate buffer.

Our results agree with the literature [32] and the electropherograms obtained using *n*-octane and *n*-heptane (data not shown) were almost identical. In the same way, when we compared the lower surface tension oils (ethyl acetate and dibutyl tartrate), we noticed no change in either selectivity or analysis time. So the discussion focused on the comparison between *n*-octane and ethyl acetate.

As expected [32], the analysis time decreased when ethyl acetate was used instead of *n*-octane. When ethyl acetate and 0.6% SDS were used, the resolution was worse than with octane, so none of the compounds separated completely. However, when we increased the SDS concentration from 0.6 to 1.2% (Fig. 2A) to enhance separation, the resolution improved but the analysis time increased slightly. Seven peaks were obtained because cloxacillin and nafcillin co-eluted and the separations between the ampicillin–penicillin G and oxacillin–penicillin V pairs were not satisfactory because the peaks overlapped.

When Brij 35 was used as surfactant, in the case of ethyl acetate, 2% had to be added because at lower concentrations, the analytes could not be separated. Clearly, the resolution of the analytes using *n*-octane (see Fig. 1C) is higher than when ethyl acetate is used (see Fig. 2B) but the analysis time dropped from around 10 to 7 min.

3.6. Effect of buffer type and concentration

The choice of buffer is extremely important in MEEKC separation. The buffer can also be used to directly affect the selectivity of a MEEKC separation. Generally, MEEKC has been performed with low-ionic strength (5–10 mM) borate or phosphate buffers [33]. These generate relatively low currents and a reasonably fast EOF. Borate was therefore chosen as the buffer to develop the method under study. Borate has so far provided fairly good results but the analytes have not been totally separated. To improve both separation and resolution, we studied increasing the amount of borate in the microemulsion mixture. We tested 10 and 20 mM borate buffer with ethyl acetate as the oil phase and Brij 35 as the surfactant. Our results were more or less the same because the resolution of the peaks was almost identical. We therefore kept 10 mM borate buffer for further analysis.

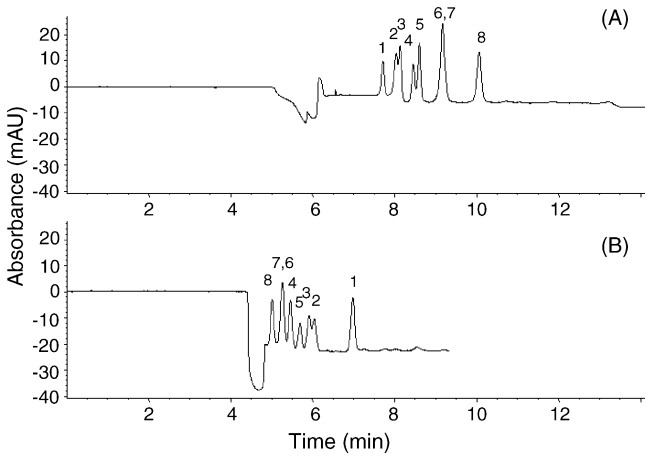


Fig. 2. Electropherograms of a standard mixture of penicillins. Separation conditions: (A) 0.5% ethyl acetate, 1.2% 1-butanol, 1.2% SDS; (B) 0.5% ethyl acetate, 1.2% 1-butanol, 2% Brij 35; in 10 mM borate buffer. Other conditions as in Fig. 1.

As we have already stated, small concentrations of borate or phosphate buffers (5–10 mM) have been widely used in microemulsion systems. However, the literature reports that zwitterionic buffers such as Tris at a concentration of 100 mM are especially useful because they generate minimum currents and create a strong EOF in which the droplets are swept towards the detector [25]. So, to increase the separation voltage and reduce analysis time, we tested Tris.

First we tested a 100 mM zwitterionic buffer, Tris, as microemulsion buffer, keeping the oil phase (0.5% ethyl acetate), the co-surfactant (1.2% 1-butanol) and the surfactant (SDS 0.6%) constant. The resolution, however, did not improve. Using the same system (Tris and SDS), we tried increasing the amount of SDS to 2% while keeping the Tris concentration constant at 100 mM. The separation clearly improved because eight peaks were observed (though six of them overlapped in pairs and the peaks were fairly asymmetrical).

Then we studied the effect of the Tris buffer with the Brij 35 system. With microemulsion systems and Brij 35 values of less than 2%, no separation was achieved. We found that the peaks were fairly asymmetrical and only seven peaks were obtained. The migration times were very similar to those of the borate system (just over 6 min).

We also tried increasing the amount of buffer, testing 130 mM Tris with 2% Brij 35. We took into account the fact that higher buffer concentrations suppress the EOF and generate high cur-

rents that may limit the level of voltage that can be applied, but in these cases, the currents obtained were too low and the peak separation did not improve.

3.7. Effect of adding organic solvents

As well as modifications to the nature and/or concentration of the co-surfactant, a more polar and water-miscible organic solvent can be added to the aqueous phase to influence its physical properties and improve the resolution [28]. These modifiers are often also described as "second co-surfactants". Using an organic solvent makes the analytes more soluble in the aqueous phase and affects electrophoretic parameters such as EOF and the electrophoretic mobilities of charged analytes.

In our study, methanol (Fig. 3A), acetonitrile (Fig. 3B), isopropanol (Fig. 3C) and 2-butanol (Fig. 3D) were individually added to the microemulsion solution so that the effect of organic modifiers could be studied. The microemulsion solution consisting of ethyl acetate, 1-butanol, 2% Brij 35, and borate 10 mM was chosen as the optimum background electrolyte because the separation between the peaks was good and the current obtained in the analysis procedure was not as low as with Tris. The amount of organic solvent added was 10%. Fig. 3 shows the electropherograms obtained when these four organic modifiers were used and Fig. 2B shows the electropherogram obtained with no organic modifier. Our results indicate that the addition of these organic solvents increased the migration time, probably because they affect the electrolyte viscosity and slow down the EOF [22,38]. They also change the selectivity of the analytes. The separation did not improve when acetonitrile was used but with methanol, isopropanol and 2-butanol peak resolution improved. When methanol was used, all the peaks were observed, although four of them (nafcillin–cloxacillin and penicillin G–ampicillin) overlapped. However, when isopropanol and 2-butanol were used, the degree of overlapping was lower. We should point out that when the carbon number in the organic solvent increased, the resolution increased and the separation improved. The results were therefore best when 2-butanol was used as organic solvent.

In a previous study, a MEKC method for separating the same penicillin antibiotics was developed [19]. The method consisted of a 20 mM tetraborate sodium buffer and 60 mM SDS at a voltage of 15 kV at pH 8. Both this method and MEEKC present similar peak resolutions. As far as analysis time is concerned, separation by MEKC takes 17 min while separation by MEEKC takes less than 12 min but the MEEKC system requires a longer and more complex buffer preparation.

3.8. Method calibration

The optimum microemulsion solution obtained consisted of 0.5% ethyl acetate, 1.2% 1-butanol, 2% Brij 35, 10% 2-butanol and borate 10 mM. Once the method had been established, the calibration step was carried out. The linearity, range, precision and detection limit of the method were investigated essentially following International Conference of Harmonisation (ICH) guidelines. The calibration plots were found to be linear, based on external standard calibration, in the 2.5–20 mg L^{-1} range for penicillin G and ampicillin and in the 1–20 mg L^{-1} range for the other compounds. In order to draw the slope, five standard solutions were prepared in this range and three points were made at each level. The correlation coefficients (R^2) were satisfactory and the detection limits, based on a signal-to-noise ratio of 3, ranged from 0.5 to 1 mg L^{-1} (see Table 2).

Multiple inter-day and intra-day injections of several solutions of all penicillins were performed to verify the intermediate precision and repeatability of the peak area. The intermediate precision was investigated by injecting a solution containing the eight penicillins at a concentration of 5 mg L^{-1} on four different days. The repeatability was calculated by analyzing the same concentration standard four times in the same day. The relative standard deviation, R.S.D., was calculated in terms of the

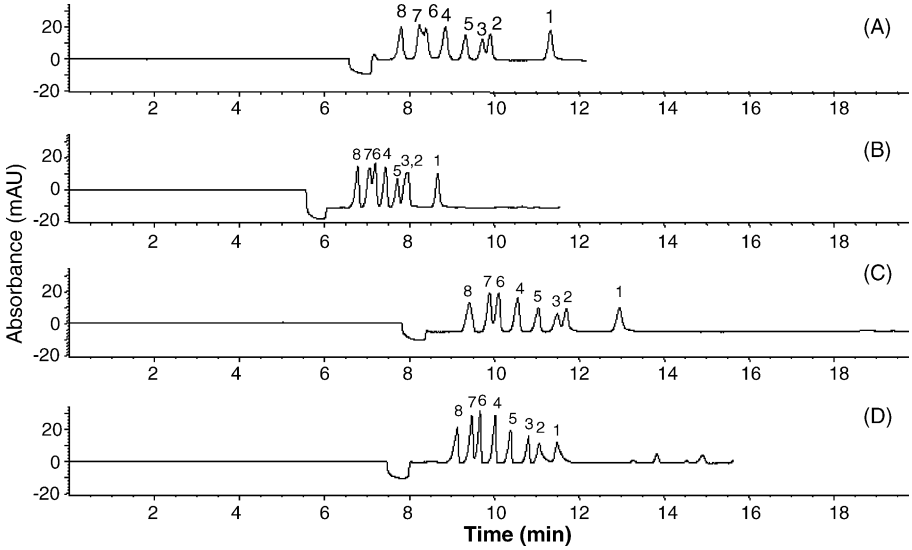


Fig. 3. Electropherograms of a standard mixture of penicillins. Separation conditions: 0.5% ethyl acetate, 1.2% 1-butanol, 2% Brij 35 in 10 mM borate buffer and (A) 10% methanol; (B) 10% acetonitrile, (C) 10% isopropanol and (D) 10% 2-butanol. Other conditions as in Fig. 1.

Table 2
Linearity, calibration curves, repeatability and intermediate precision (R.S.D.) and detection limits (LODs) of the method for standard solutions

Compound	Linearity (mg L^{-1})	Calibration curves	R^2	% R.S.D.[a] ($n = 4$)	% R.S.D.[b] ($n = 4$)	LODs (mg L^{-1})
Amoxicillin	1–20	$y = 3.4934x - 2.6518$	0.99	4.4	8.5	0.5
Ampicillin	2.5–20	$y = 2.8623x - 1.5195$	1.00	8.0	8.3	1.0
Penicillin G	2.5–20	$y = 1.3882x - 0.564$	0.99	9.0	10.5	1.0
Oxacillin	1–20	$y = 4.166x - 0.9834$	1.00	4.9	5.1	0.5
Penicillin V	1–20	$y = 2.7636x - 0.4938$	0.99	5.6	8.6	0.5
Cloxacillin	1–20	$y = 4.5489x - 1.1656$	1.00	3.6	5.0	0.5
Nafcillin	1–20	$y = 4.7209x - 0.9861$	1.00	3.5	3.9	0.5
Dicloxacillin	1–20	$y = 4.6256x - 2.0261$	0.99	3.0	3.4	0.5

Other CE conditions as in Fig. 3D.
[a] R.S.D. in terms of intra-day precision at 5 mg L^{-1}.
[b] R.S.D. in terms of inter-day precision at 5 mg L^{-1}.

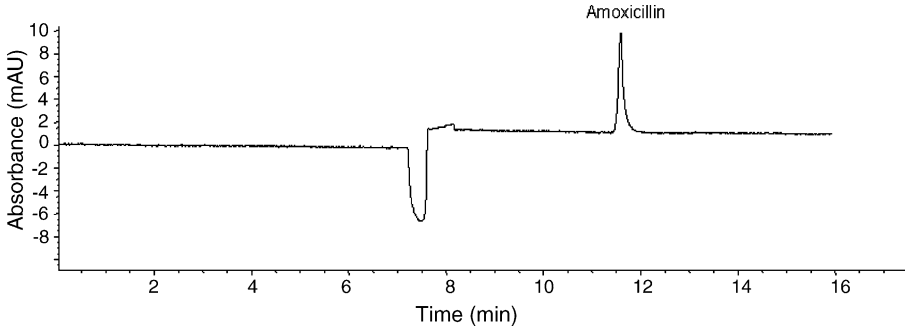


Fig. 4. Electropherogram of the commercial pharmaceutical sample of amoxicillin at a concentration of 10 mg L^{-1}. CE conditions as in Fig. 1.

peak area. The R.S.D.s, obtained at the level of 5 mg L^{-1} for all analytes, are summarized in Table 2.

3.9. Application to commercial drugs

The potential of this method for analyzing a real sample was demonstrated by determining amoxicillin in pharmaceutical samples. A commercial sample of this penicillin was assayed by the proposed method (see Fig. 4). We identified the peak observed as amoxicillin by comparing the corresponding migration time with that obtained in the standard samples (Fig. 3D). We assessed the accuracy of the method with the real sample since the composition was known, and a sample solution of 10 mg L^{-1} was analyzed. Amoxicillin was quantified at 515 mg, which is very close to the concentration of amoxicillin reported in the commercial drug (500 mg). So the recovery, defined as the percentage ratio between the determined and theoretical amounts of amoxicillin, was 103%. We also calculated the confidence interval of the method and the result was 515 ± 20 mg. These results demonstrate that the MEEKC method is suitable for determining these kinds of penicillin compounds in commercial drugs.

3.10. Enhancing sensitivity through sample stacking

As previously stated, CE is not a very sensitive technique and some applications (e.g. analysis of penicillin residues in biological fluids, animal tissues or water samples) need highly sensitive methods if these compounds are to be determined at trace levels. To enhance the detection limits, several on-column preconcentration techniques have been developed [39]. REPSM is an on-column sample preconcentration technique in which the sample, whose conductivity is lower than in the background electrolyte, is introduced into the capillary hydrodynamically and stacked by applying reverse polarity. When reverse polarity is applied after the capillary has been completely filled with the sample dissolved in distilled water, the reduced EOF presses the aqueous plug out of the capillary and into the inlet vial. The analytes in the sample plug of low conductivity get stacked at the concentration boundary, which moves backwards to the inlet. As the capillary is filled with the run buffer of high conductivity from the outlet vial, the current increases. When the current reaches 95–97% of its initial value, the polarity is changed to the separation voltage.

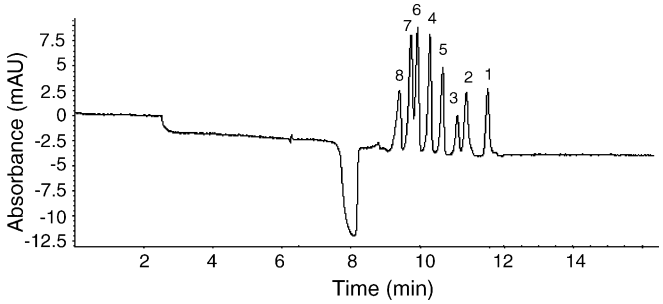


Fig. 5. Electropherogram of a standard mixture of penicillins in a concentration of 500 μg L^{-1}. Separation conditions as in Fig. 3D. Other conditions as in Fig. 1. Sample injection (REPSM): 50 mbar for 270 s, −5 kV until 95% of original current reached.

Table 3
Linearity, calibration curves, repeatability and intermediate precision (R.S.D.) and detection limits (LODs) of the REPSM method for standard solutions

Compound	Linearity ($\mu g\,L^{-1}$)	Calibration curves	R^2	% R.S.D.[a] ($n=4$)	% R.S.D.[b] ($n=4$)	Prec. fold	LODs (REPSM $\mu g\,L^{-1}$)
Amoxicillin	50–1000	$y = 0.0989x - 1.61124$	0.99	4.8	10.3	34	25
Ampicillin	25–1000	$y = 0.1052x - 0.6882$	0.98	6.1	7.1	41	10
Penicillin G	50–1000	$y = 0.0561x + 0.0997$	0.97	3.3	7.5	38	25
Oxacillin	25–1000	$y = 0.1504x + 0.2472$	0.98	2.5	5.2	37	10
Penicillin V	25–1000	$y = 0.1068x + 0.2698$	0.98	2.6	4.4	38	10
Cloxacillin	25–1000	$y = 0.1523x + 0.5487$	0.98	2.0	6.0	39	10
Nafcillin	25–1000	$y = 0.1763x + 0.3085$	0.99	3.6	6.5	40	10
Dicloxacillin	50–1000	$y = 0.1045x + 0.3665$	0.99	7.7	10.6	26	25

Other CE conditions as in Fig. 5.
[a] R.S.D. in terms of intra-day precision at 100 $\mu g\,L^{-1}$.
[b] R.S.D. in terms of inter-day precision at 100 $\mu g\,L^{-1}$.

In the present study, 50 mbar was applied to inject the sample in REPSM. We found that the time needed to completely fill the capillary with the sample was 270 s. This was accepted as the optimum time for performing the stacking step because it enabled the maximum amount of sample to be introduced into the capillary without causing peak broadening. The voltage chosen to reverse the polarity (−5 kV) was lower than the one for separation. The current dropped to 95% of the maximum current obtained with the capillary filled with the background buffer and the voltage was then switched on in order to separate the compounds. Fig. 5 shows an electropherogram of 500 $\mu g\,L^{-1}$ obtained by REPSM.

Following ICH guidelines (linearity, range, precision and detection limit), we investigated the method. Under the selected conditions, the calibration graphs were obtained by plotting the peak area against the concentration of the analyte. The data in Table 3 show that responses were linear in the sample concentration range of 25–1000 $\mu g\,L^{-1}$ except for amoxicillin, penicillin G and dicloxacillin, whose range was 50–1000 $\mu g\,L^{-1}$. As in part 3.8, the linearity of the response was investigated with five standards covering the linear range of the eight penicillins and three repetitions were made at each level. On the basis of a signal-to-noise ratio of 3 (S/N = 3), the LODs for the eight penicillins with the REPSM method were determined from a range of 10–25 $\mu g\,L^{-1}$ and enhancements in the concentration sensitivity were between 26- and 41-fold (see Table 3). The intermediate precision and repeatability were calculated in the same way as in part 3.8 but at a concentration level of 100 $\mu g\,L^{-1}$. The values are equal to or lower than 10.6% which means that the precision of the method is good.

REPSM has been previously used as a stacking technique in MEKC for analyzing the same penicillin compounds [19]. The REPSM preconcentration systems performed in MEKC and MEEKC modes both have a similar range of linearity with similar % R.S.D. in terms of inter-day precision. Moreover, although the preconcentration folds obtained were slightly higher in the MEEKC mode, the detection limits were in the same range.

4. Conclusions

Eight penicillins were successfully separated by MEEKC using a buffer solution consisting of 0.5% ethyl acetate, 1.2% 1-butanol, 2% Brij, 86.3% borate 10 mM and 10% 2-butanol at pH 10 and applying a voltage of 10 kV. The factors that most influenced the separation were the surfactant, the oil phase and the addition of organic solvents. This method allowed us to analyze a penicillin compound (amoxicillin) in a commercial pharmaceutical drug. In order to develop a more sensitive method for analyzing these compounds, we used REPSM for an on-column preconcentration of highly diluted samples. In this way, we increased sensitivity 26–40-fold and achieved LODs of between 10 and 25 $\mu g\,L^{-1}$.

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