Separation by Capillary Electrophoresis of Six Extensively Used Antibacterial Compounds

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Background: Penicillins and fluoroquinolones are two of the most extensively utilized class of antibacterial substances. Taking into account the importance of these compounds in the human and veterinary antibacterial therapy, identification and separation of these compounds in different complex matrices represent a necessity and also a challenge.
Objective: The aim of our study was to elaborate an alternative separation technique, suitable for the identification and separation of four penicillin derivatives – amoxicillin, ampicillin, benzylpenicillin and oxacillin – and two fluoroquinolones: ciprofloxacin and norfloxacin, and to optimize the analytical conditions.
Material and methods: MEKC proved to be the appropriate method of analysis for the separation of the studied compounds. The CE experiments were conducted on the Agilent 6100 CE System; the data were recorded and processed with Agilent Chemstation software.
Results: An optimum separation was achieved using a buffer solution containing 25 mM sodium tetraborate, 100 mM sodium dodecyl sulfate and 100 mM boric acid. The migration order of the six compounds was: amoxicillin, ampicillin, benzylpenicillin, oxacillin, ciprofloxacin and norfloxacin. The analytical performance of the method was evaluated by calculating the standard deviation for the peak area and also by checking the linearity of the determination.
Conclusions: The proposed method proved to be an efficient and useful tool in the separation of the studied substances and can find useful applications in the analysis of the studied substances from environmental samples.

Keywords: penicillins, fluoroquinolones, separation, capillary electrophoresis

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Introduction

Although the isolation of penicillin by Sir Alexander Fleming took place almost a century ago, the penicillin derivatives discovered and semi-synthesized in the following decades led to the development of a still extensively used class of antibiotics. Antibacterial quinolones comprise a series of antibacterial agents following the model of nalidixic acid, a naphtiridine derivative, introduced in therapy in 1963 for the treatment of urinary tract infections. As a result of extensive chemical structure – pharmacological activity relationship studies in this class, the introduction of a fluorine substituent led to the emergence of fluoroquinolones, highly potent compounds with extended activity spectrum and improved pharmacokinetic properties, which are currently used in the treatment of a variety of systemic infections. The great therapeutic importance of these derivatives are closely linked with their analytical aspects; consequently the elaboration of new methods of analysis for their identification and simultaneous separation from different matrices (environmental and biological samples) always represents a necessity and taking into account their structural similarities a permanent challenge [1,2].

The aim of our study was to elaborate a separation technique suitable for the identification and separation of four important representatives of the class betalactamin penicillins – amoxicillin, ampicillin, benzylpenicillin and oxacillin – and two fluoroquinoles: ciprofloxacin and norfloxacin. In this study, we chose one of the natural penicillins (benzylpenicillin), two semi-synthetic aminopenicillins (ampicillin, amoxicillin); a semi-synthetic izoxazolilpenicillin (oxacillin) and two frequently used structurally related flluoroquinolones. As the European Pharmacopoeia (EPh7) [3] provides high-performance liquid chromatography (HPLC) methods for their analysis, our goal is to develop an alternative method for their separation. The chemical structure of the studied antibacterials is presented in Figure 1.

Capillary electrophoresis (CE) is an officinal method in EPh7, which is gaining momentum in the analysis of pharmaceutical substances due to its speed of analysis, low

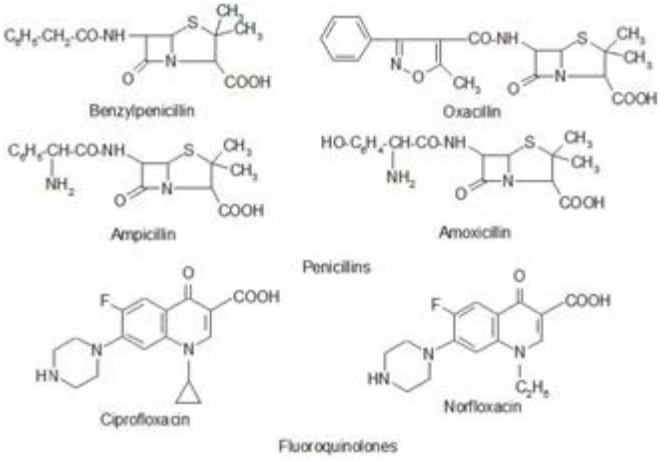

Fig. 1. The chemical structure of the studied antibacterial compounds

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consumption of samples and reagents and high selectivity, being regarded as a complementary method to the more frequently used HPLC [4,5]. The classic method of capillary zone electrophoresis (CZE) relies upon exploitation of the differences between the own electrophoretic mobilities, which are related to the charge and size of the solute.

Right from the start it was obvious that difficulties will appear at the separation of chemically related compounds like amoxicillin-ampicillin (the difference between the two compounds – a hydroxyl substituent at the terminal benzene group for amoxicillin) and especially ciprofloxacin-norfloxacin (the substituent at the nitrogen atom from the pyridin-carboxilic ring being the only difference – cyclopropyl respectively ethyl side group). Consequently to their very similar structures, these compounds exhibit very close electrophoretic mobilities, as evidenced by initial runs, and cannot be separated by CZE.

Micellar electrokinetic chromatography (MEKC) proved to be the appropriate method of analysis for the separation of the studied compounds, as the addition of a micelle-forming surfactant induces a secondary separation phase, the separation being based on the repartition of the analytes between the electrolyte solution and the micellar phase. In the separation principle there are many similarities with chromatographic methods, as addition of sodium dodecyl sulfate (SDS) to the buffer solution above its critical micellar concentration (CMC) leads to the formation of a micellar "pseudostationary" phase, which interacts with the analytes according to a partitioning mechanism; where the electroosmotic flow (EOF) acts as the chromatographic "mobile phase" [6,7,8,9,10].

Material and methods

The studied pharmaceutical substances (amoxicillin trihydrate, ampicillin trihydrate, benzylpenicillin sodium, oxacillin sodium monohydrate, ciprofloxacin hydrochloride and norfloxacin) were supplied by SC Antibiotice SA (Iași, Romania). All substances were of pharmaceutical grade.

During our experiments, the following reagents were used: boric acid, sodium tetraborate, sodium dodecyl sulfate (Merck, Germany), sodium hydroxide solution 0.1 N (Agilent). All reagents used were of analytical grade. The deionized water was prepared with a Milli-Q system (Millipore).

In the case of the penicillin derivatives and ciprofloxacin hydrochloride, we prepared 1 mg/ml stock solutions in water and later diluted to the appropriate concentrations. As norfloxacin is not soluble in water, we prepared a stock solution of 1 mg/ml in acetonitril, which we diluted with water to the appropriate concentrations. The stock solutions were stored at 2–8°C between measurements.

The experiments were conducted using the Agilent 6100 Capillary Electrophoresis System [10]; the data were recorded and processed by the Agilent Chemstation software version 7.01. In all measurements hydrodynamic injection was performed, by applying a pressure of 30 mbar for 5 seconds; the anode being at the injection end of the capillary. Separations were performed using a fused-silica capillary of 56 cm × 50 μm I.D. (effective length: 48 cm) (Agilent). The applied electrophoretic parameters: voltage +25 kV; current was kept below 200 μA and intracapillary temperature at 25°C. For the detection of the analytes we used a UV photodiode array detection system set to 210 nm and 220 nm. The pH of the buffer solutions was determined with the Terminal 740 pH–meter (Inolab). At the beginning of each day the capillary was conditioned with NaOH 0.1 N for 15 minutes and buffer solution for another 5 minutes.

Results

Different buffer solutions were tried out in order to establish the proper buffer solution. Using only a sodium tetraborate solution as running buffer, the separation of the studied compounds was not possible. Due to the similar structural characteristics, the two aminopenicillin derivatives and also the two fluoroquinolones cannot be separated by CZE. The increase of the electrolyte concentration didn't improve the efficiency of the separation, but increased migration times, due to the decrease of EOF.

Subsequent experiments were performed using a 25 mM sodium tetraborate buffer, to which sodium dodecyl sulfate was added, in gradually increasing amounts (25–100 mM). In the presence of the tensioactive substance, we achieved the separation of the two aminopenicillin derivatives, but the baseline separation of the two fluoroquinolones still remained a problem. The best results for the separation of the penicillin derivatives were obtained with a buffer solution containing 25mM sodium tetraborate and 100 mM sodium dodecyl sulfate [11].

Taking into account the fact that the dissociation of the two fluoroquinolones is strongly pH dependent, we tried to separate these substances by adding to the buffer solution increasing quantities of boric solution. In the presence of boric acid, the fluoroquinolones can be separated in the pH interval of 7.5–9. The best results were recorded at a concentration of around 100 mM boric acid in the buffer solution (Figure 2).

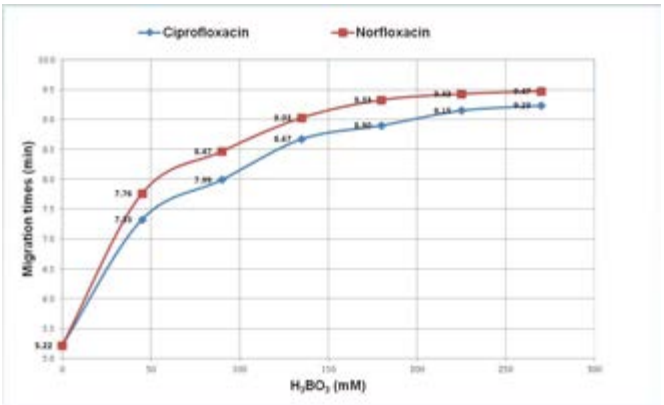

Fig. 2. Separation of ciprofloxacin and norfloxacin, depending on the concentration of boric acid

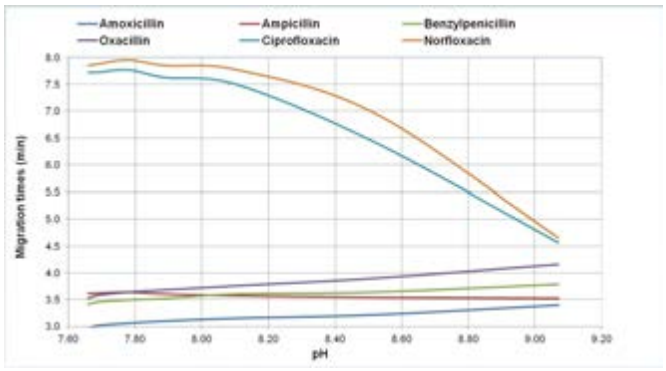

Fig. 3. The variation of the migration times of the six antibacterials, depending on the pH of the buffer solution

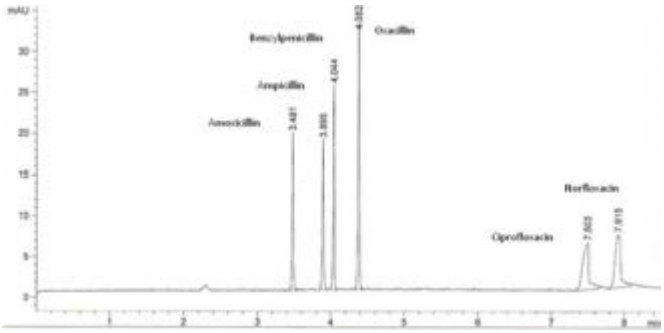

Fig. 4. The electropherogram of the separation of the six antibacterials studied

Also in the complex mixture containing all six antibacterials studied, the addition of boric acid to the buffer solution significantly improves the efficiency of the separation. The variation of the migration time depending of the pH of the buffer solution is represented in Figure 3.

In our subsequent studies we used a buffer solution containing 25 mM sodium tetraborate, 100 mM sodium dodecyl sulfate and 100 mM boric acid. In the presence of this buffer solution the six compounds can be efficiently separated, the order of separation being: amoxicillin, ampicillin, benzylpenicillin, oxacillin, ciprofloxacin and norfloxacin (Figure 4). The separation has been achieved in approximately 8 minutes.

Our aim was not only the efficient separation of the studied antibacterials, but also the optimization of the analytical conditions and understanding the separation mechanism. In order to achieve this, we studied the influence of the applied voltage and intracapillary temperature on the efficiency of the separation. The increase of the applied voltage and temperature, respectively, results in the decrease of the migration times. The modification of the injection parameters (pressure and time) does not have a significant influence on migration times. The analytical parameters chosen for the subsequent measurements were the following: applied voltage +25 kV, temperature 25°C, injection pressure 30 mbar for 5 seconds.

In order to evaluate the reproducibility of the method, we performed repeated measurements and observed the variation of the migration times, peak height and the peak area. Similar migration times, peak heights and area under curve values were obtained. We calculated the average of the values and the standard deviation; the RSD values smaller than 2% indicate that the precision of the method is good. As it is usual, the precision for migration times was better than of peak areas.

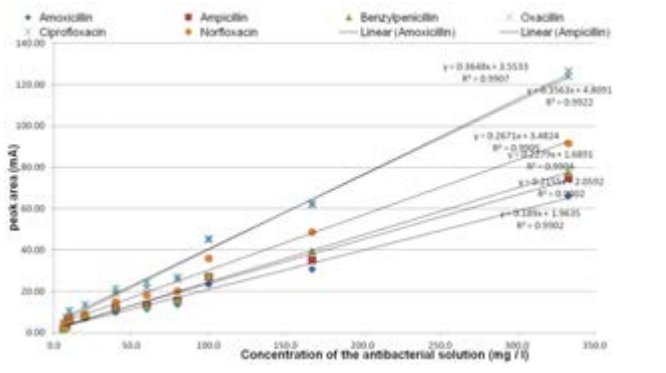

Fig. 5. The calibration curve constructed for the determination of the linearity of the method

We also calculated the individual linear regression equation and the correlation coefficient for each compound, injecting ten solutions with different concentrations in a specific range (0.5–35 mg/100 ml) and three replicates per concentration (Figure 5).

Discussions

As a rule, an acidic drug may be analyzed in its anionic form at high pH and basic drugs may be tested at low pH in their cationic form. Consequently penicillin derivatives can be separated using an alkaline buffer solution (borate buffer). Fluoroquinolones are zwitterionic drugs (containing both acidic and basic groups) and may be analyzed at either end of the pH range (phosphate or borate buffer). As our aim was the simultaneous separation of penicillins and fluoroquinolones, we chose as separation buffer a sodium tetraborate solution.

As our aim was to separate compounds with similar structural characteristics, consequently very similar electrophoretic behavior, CZE proved to be unsuitable for our purposes. MEKC can be used for the analysis of both charged and neutral analytes, extending the applicability of CE in pharmaceutical analysis.

Using a buffer solution containing 25 mM sodium tetraborate and as additives 100 mM sodium dodecyl sulfate as surfactant and 100mM boric acid, at a pH value of 8.5–9, applying a voltage of +25 kV at a temperature of 25°C, we achieved in the separation of the studied antibacterials in a time around 8 minutes.

The migration order of these compounds is governed by a combined effect of their incorporation into the hydrophobic sites of the micelles and interactions between the negatively charged micelle surface and the cationic part of the analytes. Analytes which have greater affinity for the micelle have slower migration velocities compared to analytes that spend most of their time in the bulk

phase. Penicillins are less incorporated into the micelles in comparison with the two bicyclic fluoroquinolones derivatives because of their simpler structure, therefore will migrate faster.

Some CE methods have been developed for the determination of penicillins respectively fluoroquinolones, but their simultaneous determination is challenging, because of their very similar structural and consequently physical-chemical characteristics [12,13,14,15].

Conclusions

The most efficient separation of the six studied compounds was achieved with a buffer solution containing 25 mM sodium tetraborate, 100 mM sodium dodecylsulfate and 100 mM boric acid.

The migration order of the penicillin derivatives and fluoroquinolones was the following: amoxicillin, ampicillin, benzylpenicillin, oxacillin, ciprofloxacin and norfloxacin. The influence of different analytical and electrophoretic parameters was evaluated, as well.

The analytical performance of the optimized method was evaluated on the basis of precision (by calculating regional standard deviation – RSD for the migration time and peak area), and linearity (individual regression equation and correlation coefficient).

Capillary electrophoresis proved to be an efficient tool for the simultaneous separation of the studied antibacterials, and can find future applications in the separation of these substances from environmental samples.

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