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Article

Simultaneous determination of amoxicillin and clavulanic acid in pharmaceutical preparations by capillary zone electrophoresis

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Clavulanic acid enhances the antibacterial spectrum of amoxicillin by rendering most β-lactamase producing isolates susceptible to the drug. A fast, simple and efficient capillary electrophoresis method was developed for the simultaneous determination of amoxicillin and clavulanic acid from complex mixtures. Using a 25 mM sodium tetraborate as background electrolyte at a pH of 9.30, + 25 kV applied voltage, 25 °C system temperature, UV determination at 230 nm; we succeeded in simultaneous separation of amoxicillin and clavulanic acid in approximately 2 minutes. The analytical performance of the method was evaluated in terms of reproducibility, precision, accuracy, and linearity. The optimized analytical method was applied for the determination of the two analytes from combined commercial pharmaceutical preparations. This CE method is fast, inexpensive, efficient, and environmentally friendly when compared with the more frequently used high performance liquid chromatography methods described in the literature.

Uniterms: Amoxicillin/determination. Clavulanic acid/determination. Capillary electrophoresis/quantitative analysis. Antibacterials/quantitative analysis.

O ácido clavulânico acentua o espectro antibacteriano de amoxicilina, tornando a maioria dos isolados produtores de β-lactamase sensíveis ao fármaco. Desenvolveu-se um método rápido, simples e eficiente de electroforese capilar (EC) para a determinação simultânea de amoxicilina e de ácido clavulânico a partir de misturas complexas. Usando tetraborato de sódio 25 mM como electrólito em pH de 9,30, voltagem aplicada de + 25 kV, em sistema a 25 ° C e determinação por UV a 230 nm, a foi bem-sucedida a separação simultânea de amoxicilina e ácido clavulânico em, aproximadamente, 2 minutos. O desempenho analítico do método foi avaliado em termos de reprodutibilidade, precisão, exatidão e linearidade. O método analítico otimizado foi aplicado para a determinação dos dois analitos em associação, a partir de preparações farmacêuticas comerciais. Este método de EC é rápido, barato, eficiente e ecologicamente correto, quando comparado aos métodos de cromatografia líquida de alta eficiência mais frequentemente descritos na literatura.

Unitermos: Amoxicilina/determinação. Acido clavulânico/determinação. Eletroforese capilar/análise quantitativa. Antibacterianos/análise quantitativa.

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INTRODUCTION

Amoxicillin (AMX), (2*S*,5*R*,6*R*)-6-{[(2*R*)-2-amino-2-(4-hydroxyphenyl)-acetyl]amino}-3,3-dimethyl-7-oxo-4-thia-1-azabicyclo[3.2.0]heptane-24-carboxylic acid, is a β-lactam semisynthetic penicillin from the aminopenicillin class with a broad antibacterial spectrum, used to treat a large number of infections with susceptible Gram-positive and Gram-negative bacteria. It is one of the most frequently prescribed penicillin derivatives within the class because it is better absorbed, following oral administration, than other β-lactam antibiotics (Block, Beale, 2011).

AMX is susceptible to degradation by β-lactamase producing bacteria, which are resistant to a narrow spectrum of β-lactam antibiotics, such as natural penicillins. For this reason, it is often combined with clavulanic acid, a β-lactamase inhibitor.

Clavulanic acid (CLA), (2*R*,5*R*,*S*)-3-(2-hydroxyethylidene)-7-oxo-4-oxa-1-aza-bicyclo[3.2.0] heptane-2-carboxylic acid, is an oxapenam derivative lacking the 6-acylamino side chain characteristic for penicillin derivatives, which exhibits very weak antibacterial activity, and, therefore, is not useful as an antibiotic. It is used combined with penicillin group antibiotics to overcome resistance to bacteria that secrete β-lactamase. CLA can be described as a "suicide inhibitor", covalently bonding to a serine residue in the active site of the β-lactamase (Block, Beale, 2011).

Combining these two drugs increases effectiveness by reducing susceptibility to β-lactamase resistance. Combinations of AMX trihydrate and the potassium salt of CLA are available in various fixed-doses of oral and injectable dosage forms intended for the treatment of skin, respiratory, ear, and urinary tract infections caused by β-lactamase producing bacterial strains (Block, Beale, 2011; Todd, Benfield, 1990).

The chemical structures of AMX and CLA are presented in Figure 1.

Taking into consideration the fact that the combination AMX – CLA is frequently prescribed for many infections caused by susceptible bacteria, the development of new modern analytical methods for the simultaneous determination of the two substances is a permanent necessity and also due to their particular physicochemical and structural characteristics, a challenge.

Usually, high performance liquid chromatographic (HPLC) methods are used for the simultaneous determination of the two substances from pharmaceutical products (Tai-Li *et al.*, 1997; Aghazedeh, Kazemifard, 2001; Pajchel, Pawlowski, Tyski, 2002) and human plasma (Hoizey *et al*., 2002; Kyung-Hwan *et al*., 2004; Foroutan *et al*., 2007), using different special techniques such β-cyclodextrin as stationary phase (Tai-Li *et al*., 1997), reversed phase RP – HPLC (Foroutan *et al*., 2007), HPLC - electrospray ionization (ESI) - mass spectrometry (MS) (Kyung-Hwan *et al*., 2004), with amperometric detection (Aghazedeh, Kazemifard, 2001) or UV detection (Pajchel, Pawlowski, Tyski, 2002; Hoizey *et al*., 2002).

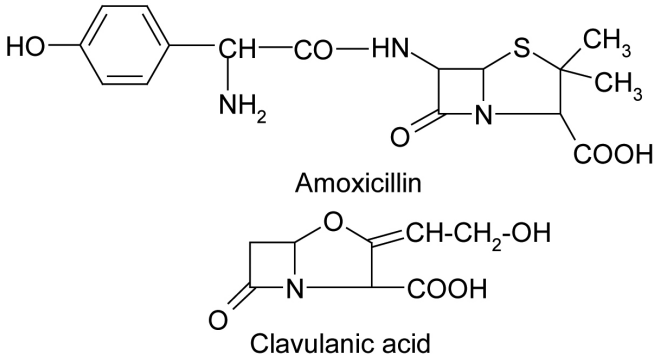

FIGURE 1 - Chemical structures of AMX and CLA.

Simultaneous determination of these compounds in complex preparations have also been carried out by UV spectrophotometric measurements using 1st order derivative spectrophotometry (Abdel-Moety *et al*., 1999; Bobrowska-Grzesik, 2001).

Surprisingly, only one capillary electrophoresis (CE) method with UV detection has been reported in the literature for the simultaneous determination of AMX and CLA, as Pajchel, Pawlowski and Tyski (2002) developed a comparative study between CE and LC for simultaneous determination of amoxicillin/clavulanic acid and ampicillin/sulbactam in pharmaceutical formulations for injections. This latter method uses a phosphate–borate buffer containing 14.4% sodium dodecyl sulfate at pH 8.6 () for the separation.

Nevertheless, CE is frequently used in the quantitative and qualitative determinations of different β-lactam antibiotics being considered today a useful alternative and also a complementary technique to the more frequently used HPLC methods (Garcia-Ruiz, Marina, 2006; Baillon-Perez *et al*., 2009; Garcia-Campana *et al*., 2009).

Our aim was the development of a new alternative CE method for the simultaneous separation of AMX and CLA, the optimization of the electrophoretic parameters and also to verify the applicability of the newly developed method in the determination of the two β-lactam derivatives from pharmaceutical preparations.

MATERIAL AND METHOD

Instrumentation

CE determinations were performed on an Agilent 7100 CE system (Agilent, Waldbronn, Germany) equipped with a DAD detector. Data were collected using Chemstation 7.01 (Agilent, Waldbronn, Germany) software. Separations were performed using a short 28 cm length (20 cm effective length) x 50 μm I.D silica-fused capillary (Agilent, Waldbronn, Germany). pH measurements were performed on a Terminal 740 pH meter (Inolab, Germany). In order to establish the optimum wavelength for the CE determination, we previously recorded the UV spectrum of the two substances using a Specord 210 UV-VIS spectrophotometer (Analytik Jena, Germany).

Chemicals and reagents

Amoxicillin trihydrate and potassium clavulanate were supplied by Antibiotice (Iaşi, Romania). All reagents: methanol, sodiumtetraborate, sodiumhydroxide (Merck, Darmstadt, Germany) were of analytical grade. Purified water was provided by a Milli-Q Plus water purification system (Millipore, USA).

For the determination from commercial pharmaceutical preparations we used Augmentin® tablets (Glaxo Wellcome, UK) each tablet containing 500 mg AMX and 125 mg CLA, obtained from a local pharmacy.

Sample preparation

Standard stock solutions of the analytes in a concentration of 1 mg mL^{-1} were prepared in water and later diluted to the appropiate concentration. All samples and buffers were filtered through a 0.45 μm syringe filter and degassed by ultrasound for 5 minutes before use. The electrophoretic runs were performed as quickly as possible, due to the instability of β-lactams in solution. The samples were introduced in the system at the anodic end of the capillary by hydrodynamic injection.

For the analysis of pharmaceutical preparations 10 Augmentin® tablets were weighed accurately and the average mass of one tablet was calculated. Tablets were pulverized and an average weight of a single tablet, equivalent to 500 mg amoxycillin and 125 mg clavulanic acid was weighed, transferred to a 100 mL volumetric flask and diluted with water to the mark. The content of the flask was ultrasonicated for 10 minutes, and the solution was filtered through a 0.45 μm syringe filter. 1 mL filtrate was transferred to a 10 mL flask and diluted with water to the mark. The samples were diluted with water to suitable concentrations.

Synthetic mixtures were also prepared by adding 125 mg potassium clavulanate to 500 mg amoxycillin trihydrate, the mixture was weighed and dissolved in a 100 mL volumetric flask with water. After shaking and filtration, 1 mL filtrate was transferred to a 10 mL flask and diluted with water to the mark; the final injected amounts of AMX and CLA were equivalent to ratio of 4:1.

Electrophoretic procedure

The capillary was washed at the beginning of the day with 0.1 M sodium hydroxide for 5 minutes followed by a water wash for another 5 minutes. Before every analysis the capillary was washed for 2 minutes with the running buffer.

In the preliminary analysis we applied some "standard" electrophoretic conditions for a CE analysis: temperature 20 ˚C, applied voltage + 25 kV, injection pressure/time 50 mbar/3 sec, sample concentration 10 μg mL^{-1}. The detection wavelengths were set to 210 and 230 nm, respectively.

RESULTS AND DISCUSSION

Optimization of the analytical procedure

For the separation, we chose the simplest electrophoretic technique, capillary zone electrophoresis (CZE). In CZE, the separation mechanism is based on differences in the charge-to-mass ratio of the analytes.

An important consideration in the selection of optimum electrophoretic conditions was obtained by consulting the ionization constants and also the structural characteristics of the analytes. AMX has two pKa values; 2.8 corresponding to the – COOH group and 7.3 corresponding to the $– NH_2$ substituent; and consequently can be detected in both acid and alkaline environments; while CLA exhibits only one pKa value 2.6 corresponding to the – COOH group, and consequently will ionizes only in an alkaline environment. Therefore, for the simultaneous separation of AMX and CLA, we chose an alkaline buffer containing sodium tetraborate.

Our aim was not only the development of an efficient method for the determination of the two analytes, but also the pursuit of a systematic study regarding the influence of different analytic and electrophoretic parameters on the separation process. Efforts were focused on the optimization of the different analytical conditions (effects of buffer

concentration and pH, the presence of possible buffer modifiers) and electrophoretic parameters (applied voltage, system temperature, injection parameters), in order to obtain increased resolutions and short analysis times.

An increase in buffer concentration modified only on the migration times of the analytes, but had only a slight effect on the resolution of the separation. The higher the buffer concentration, the later the migration time of the analytes, because increasing the concentration of the electrolyte decreases the electroosmotic flow (EOF). The optimum buffer concentration was set to 25 mM.

The pH of the buffer is the main factor affecting resolution. The pH was adjusted by adding 0.1M NaOH solutions to the original buffer solution. Migration times had the tendency to increase at high pH values, but the resolution became poor. The optimum pH value for the separation was set to 9.30.

Electrophoretic velocities of the analytes are directly proportional to field strength, so the use of high voltages will result in shorter analysis time, the limiting factor being the Joule heating, which can cause the destabilization of the electrophoretic system. As temperature increases, the viscosity of the electrolyte decreases, thus the electrophoretic mobility of the analytes increases as well. The optimum voltage was set to +25 kV while the optimum temperature was set to 25 °C, in order to obtain good resolutions and a short analysis times.

The injection parameters influence mainly the shape and amplitude of the peaks, consequently, a high injection pressure of 50 mbar and a short injection time of 1 second provided a reasonable sample load and maintained resolution.

Using buffer solution containing 25 mM sodium tetraborate, at a pH of 9.3, applying a voltage of +25 kV at a temperature of 25 °C, we achieved the simultaneous separation of the studied analytes in less than 2 minutes, the order of separation being: AMX followed by CLA (Figure 2).

Although the stability of β-lactam antibiotics in the solid state is usually satisfactory, on dissolution in water they are slowly hydrolyzed to different degradation products. Our preliminary studies using an internal standard (ciprofloxacin hydrochloride) as reference showed that within 24 hours of dissolution degradation (hydrolysis) of the two analytes was insignificant.

Analytical performances

The analytical performance of the method was evaluated in terms of reproducibility, precision, robustness, accuracy and linearity. The previously optimized CZE separation parameters were used for all these measurements.

As internal standard (IS) we used a fluoroquinolone derivative, ciprofloxacin hydrochloride; a zwitterionic compound (containing both acidic and basic groups), which can ionize in both acidic and alkaline environments. Ciprofloxacin exhibits a smaller electrophoretic mobility than the two β-lactam derivatives, and will migrate last, and also its stability in water is very good. Quantification was

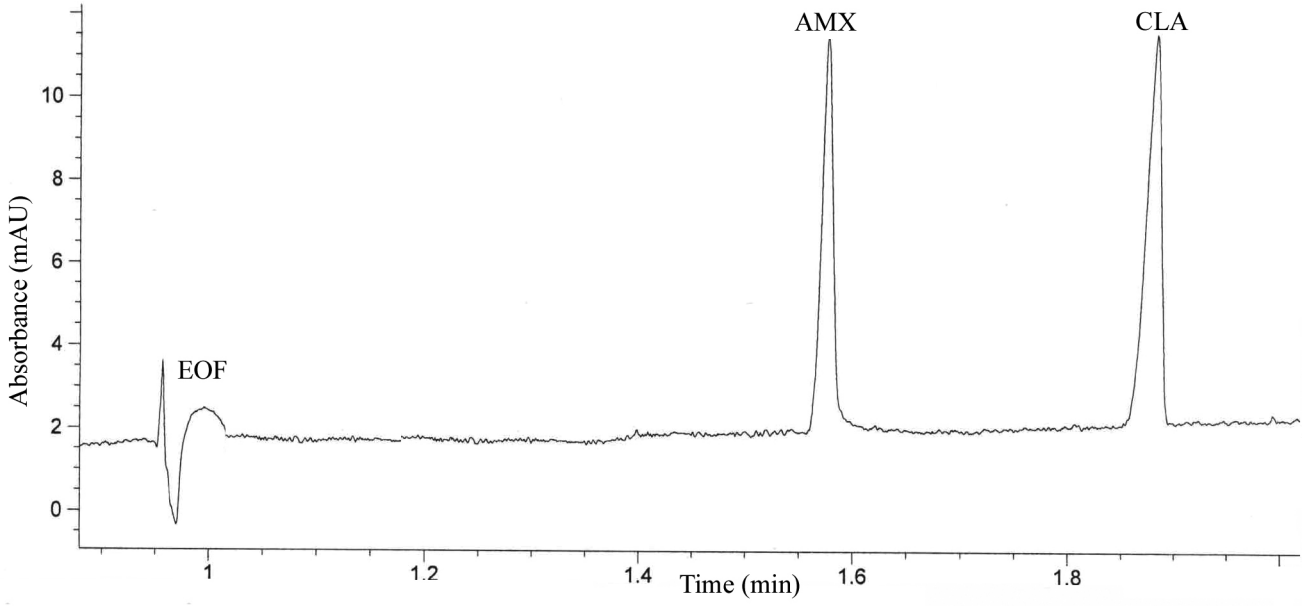

FIGURE 2 - Capillary electrophoretic separation of AMX and CLA from mixture (experimental conditions: 25 mM sodium tetraborate BGE, pH – 9.30, voltage + 25 kV, temperature 25 °C, hydrodynamic injection 50 mbar/1 sec., sample concentration 10 μg mL^{-1}, UV detection 230 nm).

accomplished on the basis of AMX respectively CLA to IS peak-area ratios (peak area of AMX-CLA/peak area of IS).

The intra-day (average of 6 consecutive measurements taken on the same day) and inter-day precision (average of 6 measurements during two days) at three different concentrations (5, 10, 20 μg mL^{-1}) was also determined. Precision, expressed as relative standard deviations (RSD%), was high with stable migration times and good repeatability of peak areas for both analytes. Very similar migration times and peak areas were obtained; the RSD values of the migration times were smaller than 1%, while the precision of the response was slightly worse (but smaller than 2.5%). The day to day analyses showed better repeatability for AMX in comparison with CLA (Table I).

The individual linear regression equations for AMX and CLA were calculated according to six concentrations in a specific range (2.5 - 50 μg mL^{-1} for both analytes) and three replicates per concentration (Table I). The linear regression coefficients were always above 0.99.

The limit of detection (LOD) and limit of quantification (LOQ) were estimated as: standard deviation of regression equation/slope of the regression equation multiplied by 3.3 and 10, respectively (Table I).

The robustness of the method was examined by making slight changes to the following parameters: buffer pH (9.0-9.3), buffer concentration (25-30 mM), applied voltage (22-25 kV) and injection pressure (40-50 mbar), taking into consideration the variation of migration times. The slight variation of these parameter does not significantly modify migration times (RSD <2.5%).

The accuracy of the method was determined by using recovery experiments analyzing solutions of known concentrations within the linearity range at three levels (10, 25 and 50 μg mL^{-1}), the mean recovery was 99.10% for CLA acid and 99.80% for AMX (n = 3 at each concentration level), respectively.

Specificity of the method was confirmed by addition of AMX and CLA reference substances to an Augmentin sample solution, only two distinct properly separated peaks were obtained.

The optimized procedure was applied to the analysis of AMX and CLA found in combined pharmaceutical preparations. Ten samples prepared from pharmaceutical formulations were analyzed, and three injections were done to obtain the average values of drug concentration. All the label claims were in the range of 95.5–101.5%, and the results were in agreement with the contents declared by the manufactures (Table II).

The peaks obtained from the samples prepared from tablets were very similar to those obtained from standard and there were no noticeable interferences from the matrix. It is important that tablet excipients do not interfere in the determination of the studied analytes, since they allow direct injections, thus involving minimum handling.

TABLE I - Analytical performance of the CZE separation

Parameter	Amoxicillin	Clavulanic acid
Day by day repeatability		
RSD migration time (%)	0.33	0.41
RSD peak area (%)	0.97	1.12
Day to day repeatability		
RSD migration time (%)	0.48	0.55
RSD peak area (%)	1.44	1.97
Linearity		
Regression equation	y= 0,474x + 0,9406	y= 0,5057x + 1,2639
Correlation coefficient	0.9980	0.9950
LOD (μg mL^{-1})	2.87	2.95
LOQ (μg mL^{-1})	8.69	8.95

TABLE II - Determination of active compounds from Augmentin 625 mg (500 mg AMX + 125 mg CLA)

Substance	Declared quantities (mg)	Found quantities (mg)	RSD (%)	SD (%)
AMX	500	496.44	0.38	0.92
CLA	125	123.35	0.24	1.88

RSD – relative standard deviation, SD – standard deviation

CONCLUSION

Differences between electrophoretic behavior and mobility of AMX and CLA allow good separation and precise, simultaneous determination of the studied analytes using a simple and rapid CZE method.

There are a number of reports on simultaneous determination of AMX and CLA by HPLC methods, but, although these methods are selective and sensitive, they are not the most suitable for routine analytical analysis because of their special requirements and financial reasons. Compared with these previously published methods the advantages of our CE method are related to: fast analysis time, rapid method development, and low consumption of analytes and organic solvents.

The proposed technique can be successfully used for the simultaneous determination of AMX and CLA from combined pharmaceutical preparations.

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