

# Application of capillary electrophoresis to the simultaneous determination and stability study of four extensively used penicillin derivatives

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The applicability of capillary electrophoresis for the analysis of four extensively used penicillin derivatives (benzylpenicillin, ampicillin, amoxicillin, oxacillin) has been studied. Because of structural similarities, the electrophoretic behavior of these derivatives is very similar; consequently an efficient separation using the conventional capillary zone electrophoresis is hard to be achieved. Their simultaneous separation was solved by using micellar electrokinetic capillary chromatography, the separation being based on the differential partition of the analytes between the micellar and aqueous phase. Using a buffer solution containing 25 mM sodium tetraborate and 100 mM sodium dodecyl sulfate as surfactant, 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 penicillin derivatives in less than 5 minutes. The separation conditions were optimized and the analytical performance of the method was evaluated in terms of precision, linearity, limit of detection, and quantification. Also, a simple capillary zone electrophoresis method was applied to study the stability of the studied penicillin derivatives in water at different temperatures, using ciprofloxacin hydrochloride as internal standard. It was observed that the extent of the hydrolysis of penicillins in water is highly dependent on the time and also temperature.

**Uniterms:** Penicillin/derivatives/stability study. Capillary electrophoresis/drugs analysis.

Estudou-se a aplicabilidade de electroforese capilar para a análise de quatro derivados de penicilina (benzilpenicilina, ampicilina, amoxicilina, oxacilina) amplamente utilizados. Em razão das semelhanças estruturais, o comportamento electroforético destes derivados é muito semelhante e, por conseguinte, a separação eficaz utilizando a electroforese capilar de zona convencional é difícil de ser efetuada. A separação simultânea foi realizada por cromatografia capilar electrocinética micelar, que se baseia na partição diferencial entre os analitos na fase micelar e aquosa. Utilizando-se solução tampão contendo 25 mM de tetraborato de sódio e 100 mM de dodecil sulfato de sódio, como agente tensioativo, com pH de 9,3, voltagem de +25 kV, à temperatura de 25 °C, obteve-se a separação simultânea das penicilinas estudadas em menos de 5 minutos. As condições de separação foram otimizadas e o desempenho do método analítico foi avaliado em termos de precisão, linearidade, limite de detecção e de quantificação. Além disso, aplicou-se método de electroforese capilar de zona simples para estudar a estabilidade de penicilinas em água a diferentes temperaturas, utilizando cloridrato de ciprofloxacino como padrão interno. Estabeleceu-se que o grau de hidrólise de penicilinas em água é altamente dependente do tempo e também da temperatura.

**Unitermos:** Penicilina/derivados/estudos da estabilidade. Electroforese capilar/ análise de fármacos.

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## INTRODUCTION

Penicillin derivatives are still among the most widely used antibiotics worldwide, as they are generally well tolerated, apart from hypersensitivity reactions, and are usually bactericidal due to their inhibitory action on the synthesis of the bacterial cell wall, but increased resistance has limited their use (Sweetman, 2011).

A mixture of penicillins is rarely administered simultaneously, but complex mixtures are often found in environmental samples (water, soil). The extensive use of penicillins causes their presence in environment and food products of animal origin, which may be responsible for allergic reactions in humans and promote the occurrence of antibiotic resistant bacteria (Kummerer, 2004).

In this study we analyzed four penicillin derivatives with different structural characteristics: benzylpenicillin (PEN) – the first natural penicillin introduced in therapy; ampicillin (AMP) and amoxicillin (AMO) – two semisynthetic aminopenicillins; oxacillin (OXA) – a semisynthetic isoxazolipenicillin. The basic structure of these compounds consists of a  $\beta$ -lactamic ring fused with a thiazolidine ring. Penicillins differ from one another only by the substituent attached to the 6-aminopenicillanic acid residue. The nature of this side chain affects the antimicrobial spectrum, stability to stomach acid, and susceptibility to bacterial degradative enzymes (Block, Beale, 2004).

The chemical structures of the studied penicillins are presented in Table I.

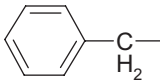
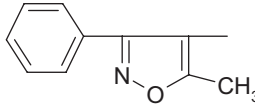
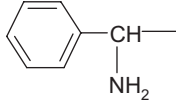
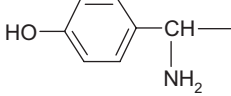
The great therapeutic importance of penicillins is closely linked to their analytical aspects; consequently, elaboration of new analytical methods for their analysis is always a necessity and also a permanent challenge.

Capillary electrophoresis (CE) is an official method in the European Pharmacopoeia 7 (2011), comprising a family of related techniques that employ narrow-bore silica capillaries; the separation being facilitated by the use of high voltage, which generates electroosmotic and electrophoretic flow of the buffer solution and ionic analytes, respectively, within the capillary.

Because of its separation efficiency, low amount of sample and reagents consumption, speed of analysis and applications to a wider selection of analytes, CE is gaining momentum in the analysis of pharmaceutical substances, being regarded nowadays as an alternative and also a complementary method for the more frequently used high performance liquid chromatography (HPLC) methods (Ahuja, Jimidar, 2008; Landers, 2008).

In recent years, quite a few CE studies on the determination of penicillins by means of capillary zone

**TABLE I** - The chemical structure of the studied penicillins

Penicillin derivative	R
Benzylpenicillin	
Oxacillin	
Ampicillin	
Amoxicillin	

electrophoresis (CZE) (Pajchel, Michalska, Tyski, 2005; Yongxin *et al.*, 1997a) and micellar electrokinetic capillary chromatography (MEKC) (Bailon Perez, Cuadros Rodriguez, Crusses Blanco, 2007; Nozal, Arce, Rios, 2004; Yongxin *et al.*, 1997b) have been published. Usually CZE has been used to analyze a single compound and/or its metabolite(s), while MEKC was the method applied for the simultaneous determination of several penicillins (Hernandez, Borrull, Calull, 2003; Nozal, Arce, Rios, 2004; Bailon Perez, Cuadros Rodriguez, Crusses Blanco, 2007; Tian *et al.*, 2011).

Also MEKC can be especially useful for the determination of penicillins from samples having high protein content (clinical samples, biological fluids), reducing the disadvantageous matrix effects caused by organic materials, while CZE through its simplicity and versatility can be successfully used for the determination of penicillins from pharmaceutical formulations (Hernandez, Borrull, Calull, 2003; Puig *et al.*, 2005). CE methods for the specific penicillin residues determination from environmental (Bailon Perez *et al.*, 2009) and food (Kowalski, Konieczna, 2007; Bailon Perez *et al.*, 2009) samples were also reported lately.

Due to common structural characteristics, the studied penicillins exhibit very similar electrophoretic mobilities, therefore an efficient separation by a conventional CZE (method based on the differences between the own electrophoretic mobilities of the analytes) cannot be

achieved (Yongxin *et al.*, 1997 a). From the beginning, it was clear that the separation of the two aminopenicillins would raise difficulties, as the only difference between AMO and AMP is the presence of the hydroxyl group attached to the aromatic ring in the side chain.

Their separation can be solved by using MEKC, where surfactants are added to the buffer solution in a concentration above their critical micellar concentration (CMC) in order to form micelles, the separation being based on the partition of the analytes between the micellar and aqueous phase (Nozal, Arce, Rios, 2004; Landers 2008).

The aim of our work was the elaboration of a simple, rapid and efficient CE method for the separation of penicillins from a complex mixture, and also to study the stability in solution of these antibiotics, depending on time and temperature.

## MATERIAL AND METHOD

The studied compounds (amoxicillin trihydrate, ampicillin trihydrate, benzylpenicillin sodium, oxacillin sodium monohydrate) were supplied by Antibiotice Iași, Romania. All the substances were of pharmaceutical grade.

Reagents of analytical grade were obtained from various distributors: sodium tetraborate, sodium dodecyl sulfate (Merck, Germany), phosphoric acid (Fluka, Germany), sodium hydroxide solution 0.1 N (Agilent). Deionized water was prepared with a Milli-Q system (Millipore).

We conducted our experiments on Agilent 6100 CE system. The electropherograms were recorded and processed by Chemstation 7.01 (Agilent). The pH of the buffer solutions was determined with the Terminal 740 pH-meter (Inolab). The samples were introduced in the system at the anodic end of the capillary by hydrodynamic injection. Separations were performed using polyimide-coated fused silica-capillaries of 56 cm (effective length: 48 cm) x 50  $\mu\text{m}$  I.D. (Agilent).

The sample solutions were prepared by dissolving solid salts in water just before the analyses. The electrophoretic runs were performed as quickly as

possible, due to the instability of penicillins in solution.

The capillaries were preconditioned with 0.1 M NaOH (2 min), distilled water (2 min) and buffer solution (2 min).

The detection was carried out by on-column photometric measurement at 210nm. Each component was identified from the mixture based on their individual migration time and UV spectra. Previously, we recorded the UV spectra for the studied penicillins, which are relatively similar, but definite small differences can be observed in the case of all components.

## RESULTS AND DISCUSSION

### Optimization of the separation conditions

Penicillins have acidic character (due to  $-\text{COOH}$  substituent) and are consequently ionizable in an alkaline environment. Aminopenicillins can be detected also in acid environment, due to the ionization of the  $-\text{NH}_2$  substituent, but OXA and PEN could not be detected at acid pH values. The pKa values of the studied penicillins are presented in Table II (Yongxin *et al.*, 1997 a; Hernandez, Borrull, Calull, 2003).

In the preliminary analysis we used 25 mM phosphoric acid (pH  $-2.1$ ), 25 mM disodium hydrogenophosphate  $-25$  mM sodium didydrogenophosphate (pH  $-7$ ) and 25 mM sodium tetraborate (pH  $-9.3$ ) background electrolytes (BGEs), respectively, and we also modified the pH of the buffer by adding a 0.1 M sodium hydroxide solution. We applied some "standard" electrophoretic conditions for a CE analysis: temperature  $20^\circ\text{C}$ , applied voltage  $+20$  kV, injection pressure/time 50 mbar/3 sec, sample concentration  $10\ \mu\text{g}/\text{mL}$ . After the initial runs, in order to obtain a good electrophoretic signal for all four penicillins, we chose a buffer containing sodium tetraborate (pH = 9.3), as an acid buffer can only be used for the simultaneous separation of AMP and AMO.

As we already anticipated, the use of CZE could not solve the separation of AMP – AMO, as its resolving power is based on the difference in electric charge relative to molecular size. The electric charge will depend on the

**TABLE II** - The pKa values of the studied penicillins

Penicillin	pKa <sub>1</sub>	Due to	pKa <sub>2</sub>	Due to	pKa <sub>3</sub>	Due to
AMP	2.7	-COOH	7.3	-NH <sub>2</sub>	-	-
AMO	2.4	-COOH	7.4	-NH <sub>2</sub>	9.6	-OH
OXA	2.8	-COOH	-	-	-	-
PEN	2.8	-COOH	-	-	-	-

number of carboxyl and amino groups of each analyte, respectively, but also on the pH of the buffer electrolyte, as dissociation of these groups is pH dependent.

Efforts were focused on the optimization of the analytical conditions (effects of buffer concentration and pH, the presence of possible modifiers), in order to obtain better resolutions and shorter 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 each penicillin, because the electroosmotic flow (EOF) decreases with an increase in ionic strength. The optimum buffer concentration was set at 25 mM.

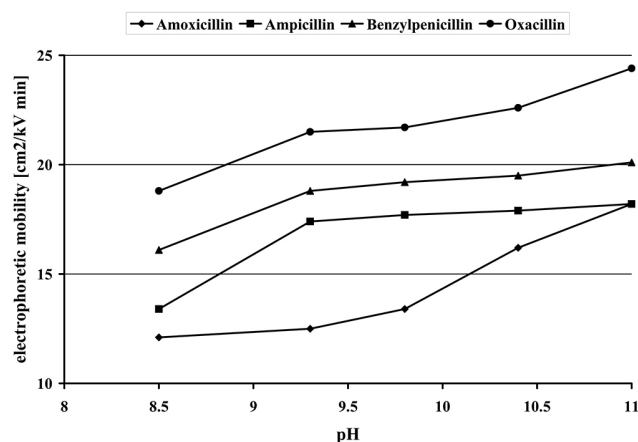
In order to improve separation, we added an anionic surfactant, sodium dodecyl sulphate (SDS), to the buffer solution. MEKC is based on a micellar “pseudostationary” phase added to the buffer solution, which interacts with the analytes according to partitioning mechanisms, in a chromatography-like mode; the EOF acting as the chromatographic “mobile phase”. The anionic SDS micelles are electrostatically attracted towards the positive electrode, but the EOF transports the bulk solution towards the negative electrode due to the negative charge on the internal surface of the silica-fused capillaries. However, the EOF is stronger than the electrophoretic mobility of the micelle under alkaline condition; therefore, the anionic micelle will travel also towards the negative electrode with a retarded velocity (Landers, 2008).

Depending on the individual partitioning equilibria of the different analytes between the micellar and the aqueous phase, a different retarding effect on the electrophoretic mobility of the analytes will be observed. The greater the percentage of the analyte distributed into the micelles, the more slowly it migrates (Silva, 2013).

The migration time increased with the increase of SDS concentration, due to the solubilization of the analytes in the micellar phase. In the presence of SDS the resolution of the separation improved considerably. The optimum surfactant concentration was set at 100 mM SDS.

The pH of the buffer is the main factor affecting resolution. The pH was adjusted by adding 0.1 M NaOH respectively 0.1 M HCl solutions to the 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 at 9.3. The influence of pH values of the background electrolyte on the effective electrophoretic mobilities of the studied penicillins is presented in Figure 1.

In order to optimize the electrophoretic conditions, we studied the influence of the applied voltage and



**FIGURE 1** - The influence of pH of the background electrolyte on the separation of penicillins (separation conditions: capillary 56 cm x 50  $\mu$ m I.D.; buffer electrolyte: 25 mM sodium tetraborate + 100 mM SDS; temperature 20 °C; applied voltage +20 kV; UV detection at 210 nm).

temperature on the separation. The increase of the voltage and temperature, respectively, results in the decrease of the migration times, but with little effect on the resolution. The optimum voltage was set at +25 kV while the optimum temperature was set at 25 °C, in order to obtain good resolutions and short analysis times.

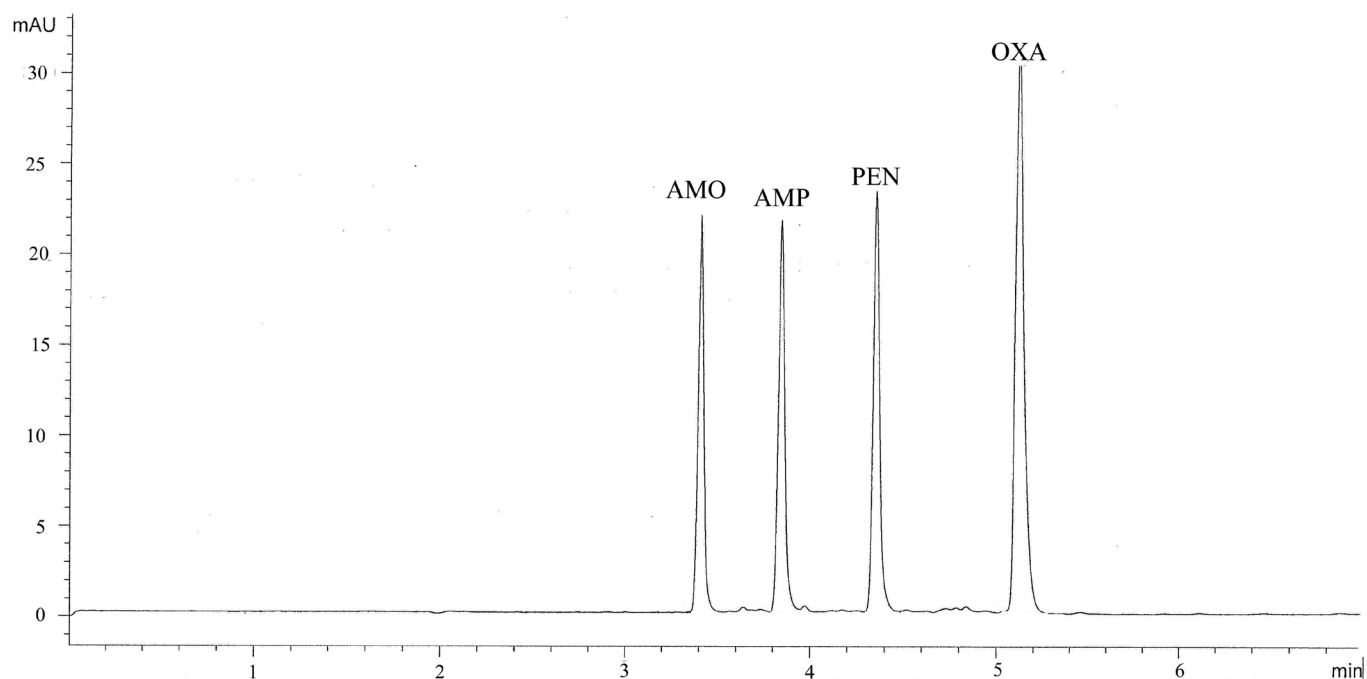
Using buffer solution containing 25 mM sodium tetraborate and 100 mM SDS, at a pH of 9.3, applying a voltage of +25 kV at a temperature of 25°C, we achieved the simultaneous separation of the studied penicillins in less than 5 minutes, the order of separation being: AMO, AMP, BENZ, OXA (Figure 2). The migration order can be explained as AMO is the more polar molecule in the mixture (because of the –OH substituent), it consequently has the lowest affinity towards micelles and migrates fastest; while OXA is the less polar molecule in the mixture (because of the large izoxazolyl substituent), it has the best affinity towards micelles and migrates last.

### Analytical performance

The optimized separation method was evaluated based on precision (migration times and peak areas), linear range, limit of detection (LD) and limit of quantification (LQ) (Table III/Table IV).

Very similar migration times and peak areas were obtained for six repeated measurements of the 4 analytes, as the RSD values were smaller than 1%. As it is usual, the precision for migration times was better than of peak areas.

The LD and LQ were calculated as the sample concentration that produces a peak signal-to-noise ratio of 3:1 and 10:1, respectively



**FIGURE 2** - Electropherogram of the separation of the 4 studied penicillins (separation conditions: capillary 56 cm x 50  $\mu$ m I.D.; buffer electrolyte: 25 mM sodium tetraborate + 100 mM SDS; pH – 9.3; temperature 25 °C; applied voltage + 25 kV; UV detection at 210 nm).

**TABLE III** - Analytical parameters of penicillins determination

Penicillin	Migration time (min)	Electrophoretic mobility ( $\text{cm}^2/\text{kV min}$ )	RSD (%) migration time	RSD (%) peak area
AMO	3.41	-13.44	0.46	0.88
AMP	3.84	-17.47	0.12	0.47
PEN	4.35	-21.5	0.20	0.63
OXA	5.12	-26.1	0.20	0.88

c=1 mg/mL, n=6

**TABLE IV** - Linearity regression data of penicillins determination

Penicillin	Correlation coefficient	LD ( $\mu\text{g/ml}$ )	LQ ( $\mu\text{g/ml}$ )	F
AMO	0.994	3.22	10.73	3.34
AMP	0.997	2.15	7.18	3.03
PEN	0.995	2.81	9.37	3.18
OXA	0.996	2.69	8.99	3.23

concentration range=1-100  $\mu\text{g/mL}$ , LD – S/N=3, LQ – S/N=10

The individual linear regression equations were calculated according to six concentrations in a specific range and three replicates per concentration. Linearity was evaluated taking into account the correlation coefficients; as correlation coefficients higher than 0.99 are considered to be evidence of good data fitting to line regression.

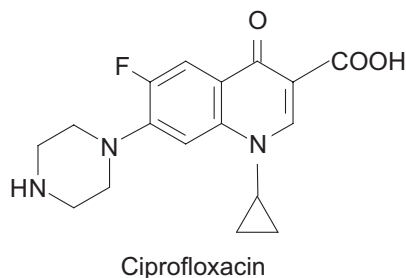
Another method to verify linearity was the application of a lack of fit ANOVA test, a statistical test, which has an F-distribution under the null hypothesis. The calculated F values for the four penicillins were below the F critical value (3.58), which demonstrates a suitable linearity in the studied concentration range.

## Stability study of penicillins

The instability of penicillins, due to the presence of  $\beta$ -lactamic ring, is well known and represents a controversial problem. Penicillins dissolved in water undergo a rapid hydrolysis, being gradually converted to different degradation products (Block, Beale, 2004; Kummerer, 2004).

After dissolution of the penicillins in water, the sample solutions were reinjected several times over the duration of two weeks, using ciprofloxacin hydrochloride as internal standard.

Ciprofloxacin is a fluoroquinolone derivative, a zwitterionic compound, which can ionize in both acid and alkaline environment. Ciprofloxacin exhibits a smaller electrophoretic mobility than penicillin derivatives; consequently, it will migrate after the last studied penicillin. Its stability in water is relatively good (the rate of decrease over two weeks was less than 5%).

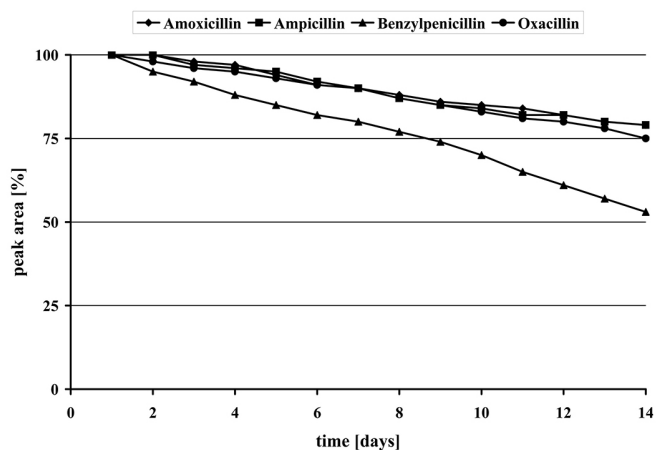


The stability of penicillins was evaluated applying a simple CZE method, using a 25 mM sodium tetraborate buffer, at a pH=9.3, a voltage of +25 kV, hydrodynamic sample injection, injection pressure/time 30 mbar/5 seconds, detection at 210 nm. We performed the separation using a short capillary of 38 cm (effective length: 30 cm) x 50  $\mu$ m I.D, in order to obtain shorter analysis times.

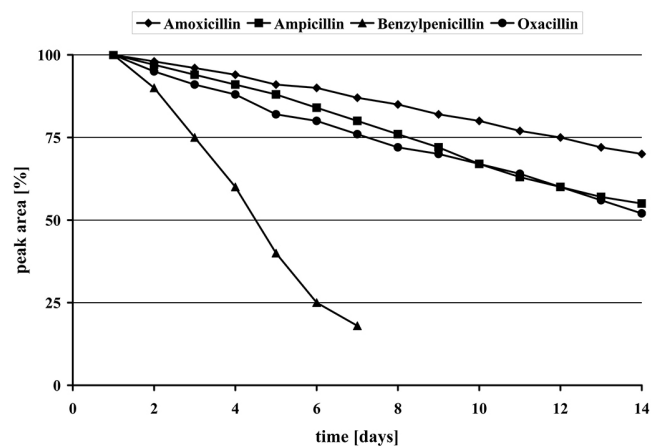
The degradation of the compounds depends highly on the temperature of the medium, with an increase of the degradation rate at higher temperatures. In our experiments, room temperature (25 °C) and refrigerator temperature (4 °C) were tested.

Figure 3 and Figure 4 present the stability diagrams of the four studied penicillins at 25 °C and 4 °C. Peaks areas obtained for the first analysis were regarded as 100%.

From the curves of Figures 3 and 4 it is obvious that the three semisynthetic penicillins (AMO, AMP, OXA) exhibit a quite similar decomposition profile, as their degradation was around 20% after two weeks at 4°C respectively 30-40% at 25 °C. PEN is by far the most unstable of the four studied substances, as its degradation



**FIGURE 3** - Solution stability diagram of the four penicillins stored at refrigerator temperature (4 °C) (separation conditions: capillary 38 cm x 50  $\mu$ m I.D.; buffer electrolyte: 25 mm sodium tetraborate; pH – 9.3, temperature 20 °C; applied voltage + 25 kV; UV detection at 210 nm).



**FIGURE 4** - Solution stability diagram of the four penicillins at room temperature (25 °C) (separation conditions: capillary 38 cm x 50  $\mu$ m I.D.; buffer electrolyte: 25 mm sodium tetraborate; pH – 9.3, temperature 20 °C; applied voltage + 25 kV; UV detection at 210 nm).

was generally around 50% at 4 °C and complete after a week at 25 °C.

At 4 °C the degradation of compounds is considerably lower in comparison with the degradation of the same compounds at 25 °C. The degradation depends also on the pH, but further investigations are necessary.

This stability investigation was facilitated by the automatic measurement repetition/ time-programming mode of the CE system.

## CONCLUSIONS

CE has proven to be an important and versatile

technique for the analysis of the investigated penicillin derivatives. The reason for studying a mixture of penicillins was to prove the applicability of CE for the analysis of penicillins in general, and for the analysis of the studied penicillins in particular. Using the optimized analytical conditions the method can be used for the analysis and identification of drugs in formulated products and also resolving separations from complex mixtures of drugs.

The stability studies showed that the extent of the hydrolysis of penicillins is highly dependent on time and temperature. The degradation rate is much smaller in the case of samples stored at lower temperatures; consequently, the samples should be stored at 4 °C and analyzed within 24 h of dissolution. This is especially important if the separation method is being applied for direct determination of penicillins from clinical and environmental samples.

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