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Short communication

Analysis of different β-lactams antibiotics in pharmaceutical preparations using micellar electrokinetic capillary chromatography

M.I. Bailón Pérez, L. Cuadros Rodríguez, C. Cruces-Blanco *

Department of Analytical Chemistry, Faculty of Sciences, University of Granada, 18071 Granada, Spain

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Abstract

The potential of micellar electrokinetic capillary chromatography (MEKC) for analyzing nine β-lactams antibiotics (cloxacillin, dicloxacillin, oxacillin, penicillin G, penicillin V, ampicillin, nafcillin, piperacillin, amoxicillin) in different pharmaceutical preparations, have been demonstrated. An experimental design strategy has been applied to optimize the main variables: pH and buffer concentration, concentration of the micellar medium, separation voltage and capillary temperature. Borate buffer (26 mM) at pH 8.5 containing 100 mM sodium dodecyl sulphate (SDS) was used as the background electrolyte. The method was validated. Linearity, limit of detection and quantitation and precision were established for each compound. The analysis of some of the β-lactams in Orbenin capsules, Britapen tables and in Veterin–Micipen injectable, all used in human and veterinary medicine, have demonstrated the applicability of these technique for quality control in the pharmaceutical industry.

Keywords: Antibiotics; Capillary electrophoresis; Pharmaceutical analysis

1. Introduction

The term "antibiotic" is normally reserved for a very diverse range of compounds, both natural and semisynthetic, which are extensively used for the treatment and prevention of diseases in humans and animals because of their antibacterial activity. Among the different classes of antibiotics (penicillins, tetracyclines, macrolides, aminoglycosides and amphenicols) [1], the β-lactams and, specially, penicillins are the most widely used. Penicillins work against bacterial infections, inhibiting the formation of the cell wall in the susceptible bacteria and can be found, as residues, in the environment and in foodstuffs but, also, the production of composed drugs, always creates a challenge for the pharmaceutical drug control.

The modern analytical investigation of antibiotic drugs, content and purity estimation of active compounds, very often involves the use of high performance liquid chromatography (HPLC) [2–5] as separation technique coupled with different detection systems.

During the last years, different methods to analyze some β-lactams antibiotics in pharmaceutical preparations using HPLC have been published [6–8]. Despite good results are obtained, this technique has some disadvantages because it requires large amounts of high purity organic solvents, long system stabilization time and special sample preparation.

Trying to resolve most of the above-mentioned problems, a relative simple technique is progressively introduced and used alternatively to HPLC [9], named capillary electrophoresis (CE). This technique [10] has become one of the most powerful separation techniques for analysis of complex mixtures from biological and biomedical fields, environmental and food sciences and even public safety, as it has been extensively discussed in numerous reviews [11–15].

Due to the fact that the basic structure of penicillins is a thiazolidizine ring connected to a β-lactam ring to which a side chain is attached, these compounds are neutral or weakly ionic molecules. For such a reason, the mode of CE most widely used to separate and analyze this type of compounds is micellar electrokinetic capillary chromatography (MEKC) using sodium dodecyl sulphate (SDS) to form the micelles, and phosphate or borate solutions as the background electrolytes (BGE). The perspectives of MEKC in drug analysis were reviewed by Nishi and Terabe [16].

* Corresponding author.
E-mail address: mcruces@ugr.es (C. Cruces-Blanco).

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In the literature, CE has not been very extensively applied to the analysis of β-lactams antibiotics. Most of the works found concerned with the application of MEKC to resolve penicillin mixtures in different matrices of environmental impact [17], or in biological samples [18,19]. The applications in pharmaceutical preparations for quality control are still even more scarce having found the work of Li et al. [20], which separated amoxicillin and 14 of its impurities in a commercial sample of amoxicillin sodium salt or the separation developed the separation of a complex mixture of 30 compounds of which 11 were β-lactams antibiotics (nine of which were penicillins) dissolved in deionized water in a MEKC system [21]. Another MEKC method was developed for the separation of ampicillin and its degradation products or the new CE analytical method evaluated and validated for determination of amoxicillin and clavulanic acid in pharmaceutical preparations [22].

In this paper, a capillary electrophoresis method with UV detection using MEKC methodology has been developed, for the first time, to separate and quantitate nine β-lactams antibiotics (cloxacillin, dicloxacillin, oxacillin, penicillin G, penicillin V, ampicillin, nafcillin, amoxicillin and piperacillin) (Fig. 1). Its applicability has been carried out with the analysis of some of these compounds in different pharmaceutical preparations, being demonstrated its used to be applied in quality control [23–27].

2. Experimental

2.1. Chemicals and stock solutions

All chemicals and solvents were of analytical reagent grade (Sigma, St. Louis, MO, USA). Sodium hydroxide were obtained from Panreac-Quimica (Madrid, Spain). Hydrochloric acid was purchased from Scharlaub (Barcelona, Spain). Sodium tetraborate, sodium dodecyl sulphate were obtained from Sigma (St. Louis, MO, USA).

Amoxicillin, ampicillin, nafcillin, cloxacillin, dicloxacillin, oxacillin, piperacillin, penicillin G, penicillin V and *p*-

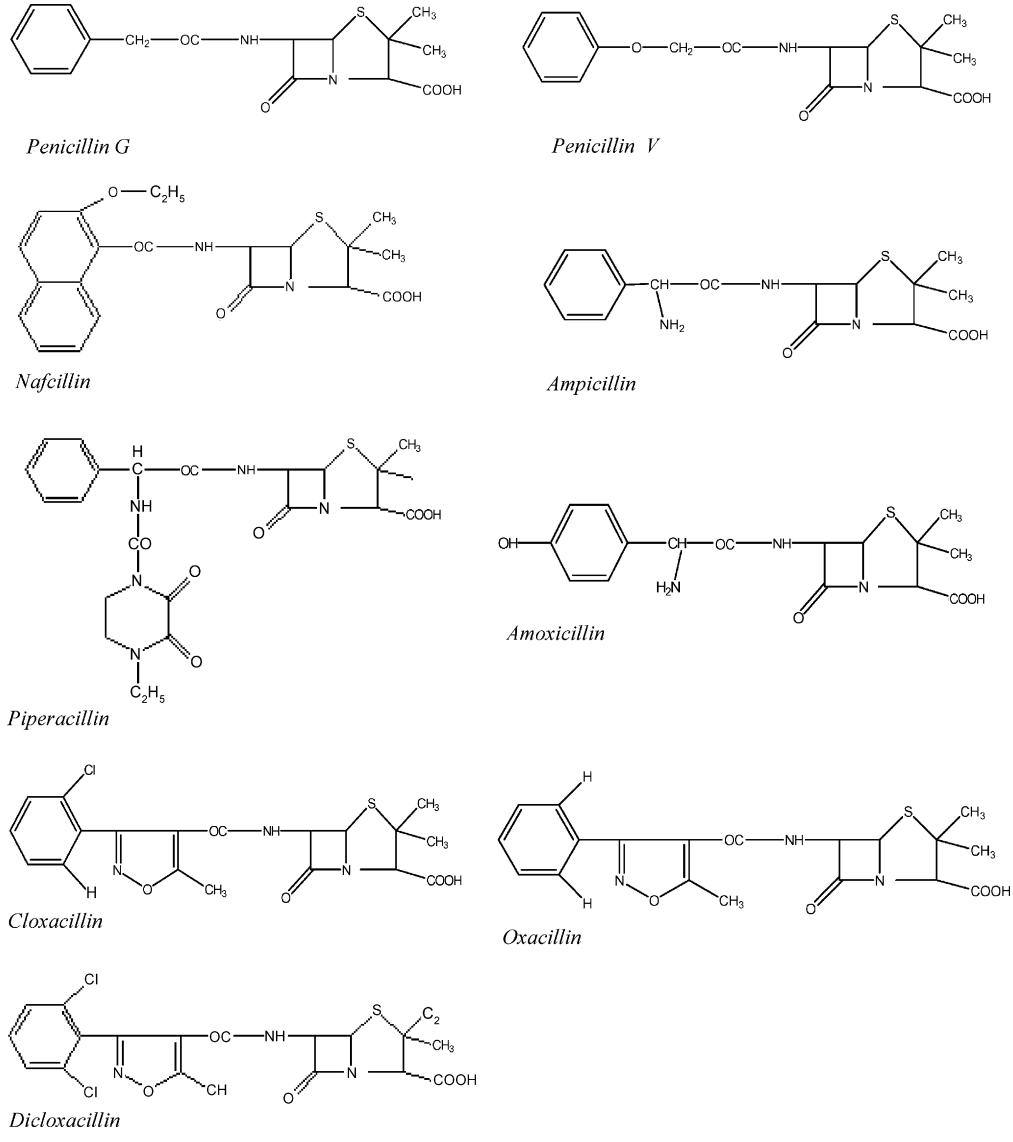

Fig. 1. Chemical structures of the studied β-lactams antibiotics.

aminobenzoic acid (PABA), used as internal standard (IS) were purchased from Sigma (St. Louis, MO, USA).

Deionized water (18.2 mΩ cm^{-1}) was prepared with a Milli-Q system (Millipore, Bedford, MA, USA).

Individual standard stock solutions of each β-lactam antibiotic and PABA (1000 μg/ml) was prepared by dissolving the appropriate amount of each substances in deionized water. The working solutions were also prepared by appropriate dilution just before use. They were stored in the dark under refrigeration and the addition of ascorbic acid (20 μg/ml) was carried out to avoid possible decomposition. Under these conditions, they were stable for, at least, 2 months.

2.2. Instrumentation and statistic software

CE experiments were carried out with a HP^{3D} CE instrument (Agilent Technologies, Waldbron, Germany) equipped with a diode-array detector. Data were collected using the software provided with the HP ChemStation, Version A.O9.01 Analytes were monitored at 220 nm. Separation was carried out in a silica fused capillary 64.5 cm × 75 μm i.d. (effective length 56 cm) in normal mode, applying a voltage of 20 kV. For pH measurements, a pH meter (Crison model pH 2000, Barcelona, Spain) was employed.

Samples injections were made in a hydrodynamic mode over 5 s under a pressure of 50 mbar. The capillary temperature was 30 °C.

When a new capillary was used, it was rinsed by passing the following solutions: H_2O Milli-Q for 5 min, 0.1 M NaOH for 5 min, H_2O Milli-Q for 5 min and finally flushed with running buffer for 15 min. Before each analysis, the capillary was flushed with NaOH for 2 min, H_2O Milli-Q for 2 min, and with running buffer for 2 min.

The electrophoretic solution was prepared by adjusting the pH of a 26 mM sodium tetraborate buffer solution with 100 mM SDS to 8.5 with 1 M hydrochloric acid. This background electrolyte was filtered before use through a 0.2 μm PTFE membrane (Millex-GN, Millipore).

For the estimation of the regression model and for the application of experimental designs in the multioptimization procedure, STATGRAPHICS was used. Peak purity evaluation was carried out by CHEMSTATION software.

2.3. Pharmaceutical analysis

Britapen tablets Reig Jofré, S.A. (Spain) and Orbenin capsules (GlaxoSmithkine, S.A., Spain) were purchased from a local drugstore while Veterin–Micipen injectable were supplied by veterinarian and are produced by Intervet, S.A. (Spain).

For the analysis of Britapen tablets, 10 units were weighed, ground in a mortar and, finally, an adequate amount of the solid was taken and dissolved in an approximate volume of 75 ml with deionized water, and dissolved by immersion in an ultrasonic bath for 20 min and filtered through a filter paper. The filtrate was diluted with deionized water to a final volume of 100 ml. For CE analysis, 12 μl of this solution was spiked with 140 μl of a *p*-aminobenzoic acid solution (1000 μg/ml) and completed with deionized water to a final volume of 10 ml, obtaining concentrations of 12 μg/ml of ampicillin and 140 μg/ml PABA.

For the analysis of Orbenin capsules, three units were taken and dissolved in an approximate volume of 75 ml with Milli-Q water and dissolved by immersion in a ultrasonic bath for 20 min and filtered through a filter paper. The filtrate was diluted with deionized water to a final volume of 100 ml. For CE analysis, 10 μl of this solution was spiked with a PABA solution (1000 μg/ml) and completed with deionized water to a final volume of 10 ml, obtaining concentrations of 5 μg/ml of cloxacillin and 14 μg/ml of PABA.

Finally, the sample preparation for Veterin–Micipen injectables, was carried out diluting the solution of an injectable in an approximate volume of 75 ml with deionized water, then dissolved by immersion in an ultrasonic bath for 15 min and filtered through a filter paper. The filtrate was diluted with deionized water to a final volume of 100 ml. For CE analysis, 18 μl of this solution was spiked with 140 μl of *p*-aminobenzoic acid (1000 μg/ml) and completed with deionized water to a final volume of 10 ml, obtaining concentrations of 18 μg/ml of penicillin G and 14 μg/ml of PABA.

3. Results and discussion

3.1. Optimization of electrophoretic variables

In order to select the best conditions and internal standard, the electrophoretic behaviour of each of the substances was studied using an experimental design strategy, which has been applied to optimize the main variables that could influence the separation and simultaneous quantification of the nine β-lactams antibiotics using the MEKC methodology: pH and concentration of the running buffer, concentration of the micellar medium, the separation voltage and the capillary temperature.

A face-centered Draper–Lin small composite design with four central points and 20 runs [28–30], scarcely applied in analytical chemistry, was selected as response surface design because of the number of levels are adequate for the variable ranges and because of their high efficiency compared with other composite design. Draper–Lin strategy reduces the number of points of the factorial design [31], obtaining a so-called "small composite design", for which a minimum experimental work is required to calculate the coefficient of the mathematical function for the response surface [31–33].

The criterion to select the optimum values for the electrophoretic variables under study was based on obtaining a maximum for a multiple response function established as a combination of the corresponding efficiencies for the nine antibiotics to obtain the best resolution of all of them in the shortest analysis time possible.

The experimental domain for the study of the selected variables was defined based on different considerations [34]. In order to predict the optimum pH range for the separation of all the β-lactams antibiotics under study, it was necessary to identify the pH values, at which the differences between their mobility was greatest.

Because it was observed that at pH values below 8, an inadequate resolution was obtained, the present design was studied varying the pH in the range from 8 to 11.

In order to predict the optimal buffer solution for carry out the separation of the compounds in these pH ranges, it was necessary to select concentration and composition of buffer at which the differences between the mobilities of the studied substances, were greatest. Buffer solutions such as borate, ammonium acetate, glicine, sodium carbonate, citrate and phosphate were proved, selecting borate buffer for giving the best results. Borate buffer concentration was modified between 20 and 150 mM in order to obtain the better resolutions of the compounds and adequate current, avoiding the Joule effect.

The addition of a micellar media to the electrophoretic buffer was taken into account because some of the drugs have very similar migration times and, therefore, it was not possible to develop a CE method with the capillary zone electrophoretic procedure (CZE) for which it was necessary to introduce a micellar media to modify the viscosity of the electrophoretic medium and, resulting in working with the MEKC methodology. The micellar medium most widely used in this type of methodology is sodium dodecyl sulphate (SDS) trying with concentrations ranging from 50 to 150 mM.

The separation voltage [35,36] was modified between 10 and 25 kV so as to obtain a good compromise between good separation and analysis time. The separation temperature was varied around room temperature to avoid temperature gradients in the capillary.

All the runs of the experimental design were carried using working solutions containing between 5 and 300 μg/ml for each β-lactam antibiotic. The matrix of experiments corresponding to this design is shown in Table 1.

From the multioptimization procedure [37–39], pH, buffer concentration and separation voltage were found significant on efficiency with optimum values of 26 mM borate buffer at pH 8.5 in presence of 100 mM SDS and carrying out the separation at 25 kV with a capillary temperature of 30 °C.

Under these conditions, a good resolution was achieved (higher than 1.5), obtaining the separation of the nine β-lactams antibiotics in 22 min. Fig. 2 shows the separation of the nine selected compounds at the optimum conditions.

Table 1
Experimental design showing the real and coded levels for all the studied variables in the optimization of the separation of nine β-lactams antibiotics under study

Runs	pH	Voltage (kV)	Buffer concentration (mM)	[SDS] (mM)
1	10.25 (1)	22.5 (1)	50 (1)	75 (−1)
2	8.75 (−1)	22.5 (1)	50 (1)	125 (1)
3	8.75 (−1)	17.5 (−1)	50 (1)	75 (−1)
4	10.25 (1)	17.5 (−1)	30 (−1)	125 (1)
5	8.75 (−1)	22.5 (1)	30 (−1)	125 (1)
6	10.25 (1)	17.5 (−1)	50 (1)	125 (1)
7	10.25 (1)	22.5 (1)	30 (−1)	75 (−1)
8	8.75 (−1)	17.5 (−1)	30 (−1)	75 (−1)
9	9.5 (0)	20 (0)	40 (0)	100 (0)
10	9.5 (0)	20 (0)	40 (0)	100 (0)
11	8.44 (−1.41)	20 (0)	40 (0)	100 (0)
12	10.56 (1.41)	20 (0)	40 (0)	100 (0)
13	9.5 (0)	16.5 (−1.41)	40 (0)	100 (0)
14	9.5 (0)	23.5 (1.41)	40 (0)	100 (0)
15	9.5 (0)	20 (0)	26 (−1.41)	100 (0)
16	9.5 (0)	20 (0)	54 (1.41)	100 (0)
17	9.5 (0)	20 (0)	40 (0)	64.75 (−1.41)
18	9.5 (0)	20 (0)	40 (0)	135.3 (1.41)
19	9.5 (0)	20 (0)	40 (0)	100 (0)
20	9.5 (0)	20 (0)	40 (0)	100 (0)

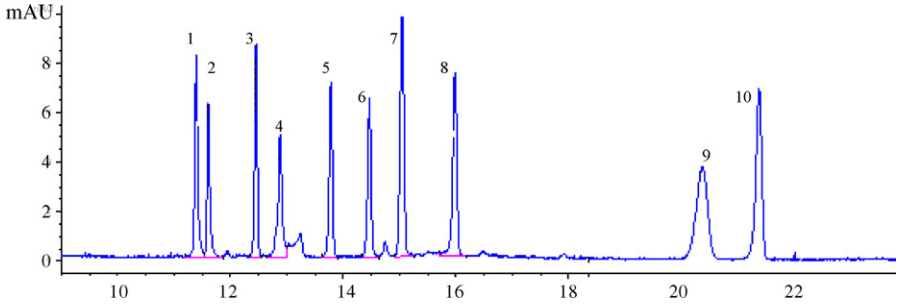

Fig. 2. MEKC separation of the nine β-lactams antibiotics and the internal standard *p*-aminobenzoic acid (PABA) under optimized conditions: pH 8.5 using 26 mM sodium tetraborate buffer containing 100 mM SDS. Separation voltage: 20 kV; capillary temperature: 30 °C. (1) ampicillin; (2) amoxicillin; (3) penicillin G; (4) piperacillin; (5) oxacillin; (6) penicillin V; (7) PABA; (8) cloxacillin; (9) nafcillin; (10) dicloxacillin.

In all cases, *p*-aminobenzoic acid (PABA) was chosen as internal standard because it is stable, commercially available in a high purity form, inexpensive, non-toxic and possessing acceptably high UV activity at the desired wavelength, giving good peak shape.

3.2. Calibration curves

Six calibration standard solutions corresponding to 1.5, 4, 8, 12, 16 and 20 mg/l were prepared for amoxicillin, 1, 5, 10, 15, 20 and 30 mg/l for ampicillin, 0.8, 2, 4, 6, 8 and 10 mg/l for cloxacillin, dicloxacillin and oxacillin, 0.8, 1, 2, 3, 4 and 5 mg/l for nafcillin, 1.5, 5, 10, 15, 20 and 30 mg/l for penicillin G and piperacillin and 1, 4, 8, 12, 16 and 20 mg/l was prepared for penicillin V; and spiked with a constant concentration of *p*-aminobenzoic acid used as internal standard (IS). Two replicates were prepared at each concentration level and each was injected in triplicate. Calibration curves were established by considering the relative corrected peak height as a function of the analyte standard concentration. Performance characteristics, calculated from the calibration data set for each compound as well as the calibration parameters obtained from the least-square regression are listed in Table 2.

The *P*-values for the lack of-fit test ($\alpha = 0.05$) show absence of curvature. The linearity (expressed as relative standard deviation (R.S.D.)) of slope was satisfactory in all cases.

3.3. Detection and quantitation limits

The limit of detection (LOD) was determined by using three times the standard deviation of the intercept divided by slope of each calibration curve. The limit of quantification (LOQ) was calculated by using 10 times the standard deviation of the intercept divided by the slope. Numerical data of the LOD and LOQ for the analyzed antibiotics are collected in Table 2.

Standard deviation of residuals was obtained by analysis of variance (ANOVA) in validation of the calibration model. The proposed methods allow β-lactams antibiotics to be determined with LODs between 0.35 and 1.42 mg/l.

The detection limits obtained in this work are low enough to determine concentrations of the studied antibiotics in numerous matrices where they could be found, such as environmental media, veterinary residues, foodstuffs or in pharmaceutical quality control.

3.4. Precision

The precision of relative corrected areas was evaluated with mixed standard solutions at three concentration levels (10, 20 and 30 mg/l of each compound) under repeatability and intermediate precision (interday) conditions. For repeatability studies, five replicate experiments were carried out on the same day. For intermediate precision, three replicate experiments were carried out on each of 5 days using the same stock buffer solution. The results, expressed as the relative standard deviation (R.S.D.) of relative corrected areas are given in Table 2. The mean migration times obtained from intermediate precision conditions was 11.3 min (1.7% R.S.D.) for ampicillin, 11.7 min (1.7% R.S.D.) for amoxicillin, 12.5 min (1.5% R.S.D.) for penicillin G, 13 min (1.6% R.S.D.) for piperacillin, 13.7 min (1.7% R.S.D.) for oxacillin, 14.5 min (1.5% R.S.D.) for penicillin V, 15 min (1.8% R.S.D.) for PABA (as internal standards), 16 min (1.7% R.S.D.) for cloxacillin, 20.2 min (1.7% R.S.D.) for nafcillin and 21.5 min (1.5% R.S.D.) for dicloxacillin, concluding that the proposed method at the studied concentrations provided acceptable precision.

3.5. Application of the proposed method in pharmaceutical preparations

Pharmaceutical containing β-lactams antibiotics both for human or animal use, are commercialized under different presentations such as capsules, tables and injections.

To demonstrate the applicability of the present methodology for the analysis of different β-lactams antibiotics in different presentations, three pharmaceutical preparations: Orbenin capsules that contains as active principle cloxacillin; Britapen tablets, whose active principle is ampicillin. These two pharmaceutical preparations are used in humans for the treatment of diverse types of infections by staphylococcus, such as mastitis, wounded and infected burns, endocarditis, meningitis, infections of the breathing tract, urinal and genito-urinal tract.

Also, the determination of another of the studied antibiotics, penicillin G, in an injectable named Veterin–Micipen which is

Table 2
Statistics and performance characteristics of the proposed method

Antibiotic	Linearity range (mg/l)	Calibration parameters			LOQ (mg/l)	LOD (mg/l)
		Intercept	Slope	R^2 (%)		
Amoxicillin	1.5–20	−0.19	0.40	99.31	2.73	0.82
Ampicillin	1–30	0.34	0.23	99.23	3.10	0.93
Cloxacillin	0.8–10	−0.28	0.73	99.27	1.18	0.36
Dicloxacillin	0.8–10	−0.10	0.62	99.14	1.27	0.38
Nafcillin	0.8–5	−0.01	0.65	99.63	1.18	0.35
Oxacillin	0.8–10	−0.14	0.65	99.11	1.23	0.37
Penicillin G	1.5–30	−0.16	0.27	99.19	3.03	0.91
Penicillin V	1–20	0.08	0.32	98.94	2.69	0.81
Piperacillin	1.5–30	−0.21	0.24	99.16	5.74	1.42

Table 3
Composition of the pharmaceutical preparations and recovery results

Presentation	Source	Composition	Values		Recovery (%)
			Nominal (μg/ml)	Found (μg/ml)	
Orbenin (capsules)	GlaxoSmithkline, S.A.	Cloxacillin (sodium salt, monohydrate); magnesium stearate	500	478	95.6
Britapen (tablets)	Reig Jofré, S.A.	Ampicillin (trihydrate); magnesium stearate; cellulose	1000	923	93
Veterin–Micipen (injections)	Intervet Laboratory, S.A.	Penicillin G (potassium salt); Penicillin G; procaine; dihydrostreptomicin sulphate	600	546	91

specially used in animals for the treatment of mastitis, metritis and pheumonia, have been carried out.

Table 3 shows the composition of the pharmaceutical preparations analyzed, their presentation, manufacturing source and the recoveries obtained using the MEKC conditions selected for each compound. The content of each analyte in the pharmaceutical formulations was determined by triplicate injections of three independently prepared solutions.

As can be observed in Table 3, the recoveries obtained for all compounds studied in the different preparations were close to 100%, with values ranging from 93 to 96%, which agree with the tolerances indicated by the USP pharmacopoeia [40]

Determinations were made in triplicate for each sample. The results obtained are summarized in Table 3, showing good results in the application of the method to the quality control of pharmaceutical preparations containing antibiotics of the β-lactam group that are used intensely in dairy farming, particularly to combat mastitis or in numerous human infections. It is important to notice that the determination of these antibiotics in the pharmaceutical preparations cited above was directly carried out without any pre-concentration step or interference from the different excipients present together with the three active compounds.

4. Conclusions

A new MEKC method with UV detection has been developed for quantitative determination of nine antibiotics of the β-lactam group, described for the first time, for its determination in different pharmaceutical preparations, demonstrating that CE is a good alternative to HPLC methods because it is simple, cheaper and offers short analysis times with very similar analytical characteristics.

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