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Determination of grepafloxacin and clinafloxacin by capillary zone electrophoresis

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Abstract

A simple and rapid capillary zone electrophoresis determination method with UV detection of grepafloxacin and clinafloxacin has been developed. The separation was performed in 35 m*M* borate–35 m*M* phosphate buffer solution (pH 8.6), containing 6% (v/v) of acetonitrile. Analyses were realised using fused-silica capillaries (57 cm length×75 μm I.D.) and the operating conditions were: 15 kV applied voltage, 30 °C and detection at 279 nm. Piromidic acid was used as an internal standard. The linear concentration range of application was 1.0–120.0 μg ml^{-1} for both compounds, with a detection limit of 0.2 μg ml^{-1} for grepafloxacin and 0.3 μg ml^{-1} for clinafloxacin. The analysis yielded good reproducibility (RSD between 3.37 and 1.74%). It was applied to the determination of grepafloxacin and clinafloxacin in human and rat urine samples. The method was validated using HPLC as a reference method. Recovery levels were between 94.5 and 103%.
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Keywords: Grepafloxacin; Clinafloxacin

1. Introduction

Fluoroquinolones have emerged as one of the most important classes of antibiotics of the past decade [1]. The newest members of this class demonstrate broad-spectrum activity against many pathogenic gram-negative and gram-positive bacteria, including many of the so-called atypical respiratory pathogens [2]. They act by inhibiting the DNA gyrase resulting in bacterial death [3].

Grepafloxacin (GREPA) {1-cyclopropyl-6-fluoro-1,4-dihydro-5-methyl-7-(3-methyl-1-piperazinyl)-4-oxoquinoline-3-carboxylic acid} and clinafloxacin (CLINA) {7-(3-amino-1-pyrrolidinyl)-8-chloro-1-cyclopropyl-6-fluoro-1,4-dihydro-4-oxo-3-quinoline carboxylic acid} are new antibacterial fluoroquinolone agents (Fig. 1), used in the treatment of patients with a variety of disorders such as pneumonia, and gynaecologic and urinary infections [4–6]. Recommended oral doses of grepafloxacin are 300–600 mg day^{-1} for 7–14 days once daily [4]. In the case of clinafloxacin, oral daily medication is ~100- or 200-mg dosages [6] twice daily.

The widespread use of these compounds and the need for clinical and pharmacological studies require

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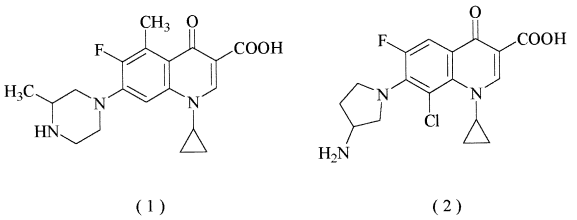

Fig. 1. Chemical structure of (1) grepafloxacin and (2) clinafloxacin.

fast and sensitive analytical techniques to determine the presence of the drugs in biological fluids. To our knowledge only high-performance liquid chromatography (HPLC) methods have been reported to determine the presence of GREPA and CLINA in biological fluids [7–12].

Capillary zone electrophoresis (CZE) is a highly efficient and less solvent-consuming technique, which separates sample components according to their sizes and charges. CZE has proved to be an excellent alternative to HPLC in drug analysis. CZE procedures exhibit greater resolution than HPLC and have the same order of precision. CZE is less costly to operate than HPLC and requires only modest quantities of the sample. UV–Vis absorbance detection in CZE has, however, a relatively poor concentration sensitivity relative to that attainable by conventional UV–Vis in HPLC.

This paper describes a CZE method for the simultaneous determination of GREPA and CLINA in human and rat urine samples.

2. Experimental

2.1. Apparatus and software

Electrophoretic measurements were performed on a Beckman (Fullerton, CA, USA) P/ACE 5500 capillary electrophoresis system equipped with a diode-array detector, a thermostated column cartridge, a high-voltage built-in power supply and an autosampler. The System Gold (version 810) software package (Beckman) was used for the acquisition and subsequent treatment of the electropherograms. Separations were carried out in an uncoated fused-silica capillary (Beckman, 57 cm×75 μm I.D.×375 μm O.D.) with a capillary to detector distance of 50 cm.

A Hewlett-Packard (Norwalk, CT, USA) 8453 UV–Vis spectrophotometer was used for recording absorbance spectra of GREPA, CLINA and piromidic acid.

HPLC was done with a chromatograph Hewlett-Packard series 1050 with UV detector set at 290 nm. The output of the detector was monitored by a chromatocorder Hewlett-Packard model number 3395A. Separation was achieved on a Lichrosphere $100RP_{18}$ column, 5-μm particle size, 125 mm×4 mm I.D. (Merck, Darmstadt, Germany). The column was protected with a pre-column Lichrocart 4-4 (10 mm×4 mm I.D.) (Merck).

Statgraphics (version 6.0) [13] and Alamin (version 1.0) [14] software packages were used for the statistical analysis of the data and regression analysis (linear model).

2.2. Reagents

All reagents were analytical-reagent grade unless stated otherwise and all solvents were HPLC grade (Panreac, Barcelona, Spain). Water was purified with a Milli-Q plus system (Millipore, Bedford, MA, USA).

Grepafloxacin was kindly provided by GlaxoWellcome (Spain) and clinafloxacin was kindly provided by Parke-Davis (Spain). Stock solutions (1.0 mg ml^{-1}) were prepared by exact weighing of reagent and dissolution in water–acetonitrile (40:60, v/v). They were stable for at least 4 weeks if stored in the dark at 4 °C. Working solutions were prepared daily by appropriate dilutions with water–acetonitrile (40:60, v/v).

Theophylline, naproxen, acetaminophen and the internal standard piromidic acid were supplied by Sigma (St. Louis, MO, USA).

The electrophoretic media was prepared daily by mixing adequate proportions of stock solutions of sodium borate (Merck) and sodium dihydrogen phosphate (Merck). After pH adjustment, the volume of acetonitrile was added.

The solutions were filtered through a 0.45-μm filter (Millipore) before use.

2.3. Electrophoretic analysis

The analyses were conducted at 15 kV and 30 °C. The detection wavelength was set at 279 nm. The samples were introduced in the anodic end of the capillary via hydrodynamic injection at 0.5 p.s.i. for 10 s. The electrophoretic media was 35 m*M* borate–35 m*M* phosphate (pH 8.6)–acetonitrile (94:6, v/v).

When a new capillary was used, it was flushed with 1.0 *M* NaOH for 10 min followed by 0.2 *M* NaOH for 10 min. Each day before use the capillary was reconditioned by flushing with 1.0 *M* NaOH for 3 min, water for 3 min, 0.2 *M* NaOH for 3 min and run buffer for 4 min. Between runs the capillary was flushed with deionised water for 3 min, 0.2 *M* NaOH for 3 min and then the run buffer for 4 min.

2.4. Sample treatment

The human urine samples were obtained from healthy volunteers. The samples were centrifuged for 10 min at 3800 rpm and filtered through a Minisart-plus syringe filter (0.2-μm pore size; Supelco, Bellefonte, PA, USA). The filtrates were collected in dark glass containers previously cleaned with nitric acid, washed with deionised water and stored at −25 °C until the analysis was performed. The urine samples were frozen to avoid microbiological degradation. Aliquots of these filtrates were diluted between 2- and 5-fold with water–acetonitrile (40:60, v/v) and treated as described in Section 2.3.

3. Results and discussion

3.1. Method development

To get the optimum capillary electrophoresis conditions, several electrophoretic media were investigated including sodium borate, sodium dihydrogen phosphate, acetate buffer, *N*-[2-hydroxyethyl]piperazine-*N′*-[2-ethanesulfonic acid] (HEPES), sodium cholate and sodium dodecil sulfate (SDS). The combined use of sodium borate–sodium dihydrogen phosphate–acetonitrile was found to be the most effective for resolving the GREPA and CLINA mixture.

The migration times of GREPA and CLINA are pH dependent. Its effect on migration time was studied in the range 8.2–9.0 with 50 m*M* borate–50 m*M* phosphate–acetonitrile (95:5, v/v) as electrophoretic media. In this range GREPA and CLINA should be negatively charged and then the electroosmotic migration velocity should be greater than the electrophoretic migration of these two compounds. Fig. 2 shows how the migration times of GREPA and CLINA increase with the solution pH. The optimum value was found at pH 8.6, the resolution being 4.72, the number of theoretical plates being then 1.5×10^4 for GREPA and 2.3×10^4 for CLINA.

The effect of the ionic strength of the buffer on the separation of analytes was studied. The total borate–phosphate buffer (1:1, v/v) concentrations were varied from 50 to 100 m*M* with 5% (v/v) acetonitrile. Fig. 3 shows increasing migration times of GREPA and CLINA when the buffer concentration increases. Results suggest that the number of theoretical plates is highest at 70 m*M*. Therefore, 70 m*M* was chosen as the optimum buffer concentration.

The optimum composition of the borate–phosphate buffer solution was established by varying the contents between 14–56 and 56–14 m*M*, respectively, finding a compromise between good resolution and peak shape with a 35 m*M* borate–35 m*M* phosphate mixture with pH 8.6.

With the above electrolyte, six different acetoni-

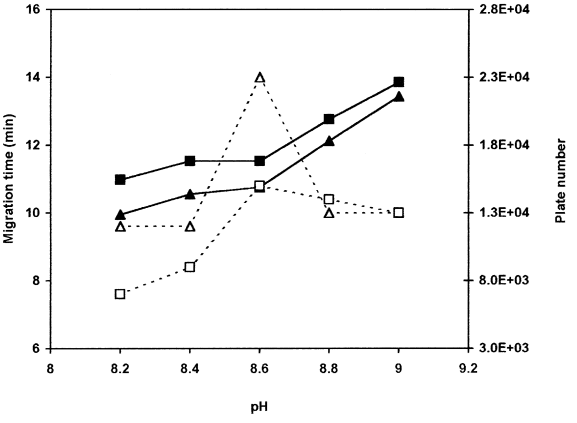

Fig. 2. Influence of pH. Buffer solution, 95% (50 m*M* borate–50 m*M* phosphate)–5% acetonitrile (v/v); 13 kV; 25 °C. Migration time: (▲) CLINA, (■) GREPA. Plate number: (△) CLINA, (□) GREPA.

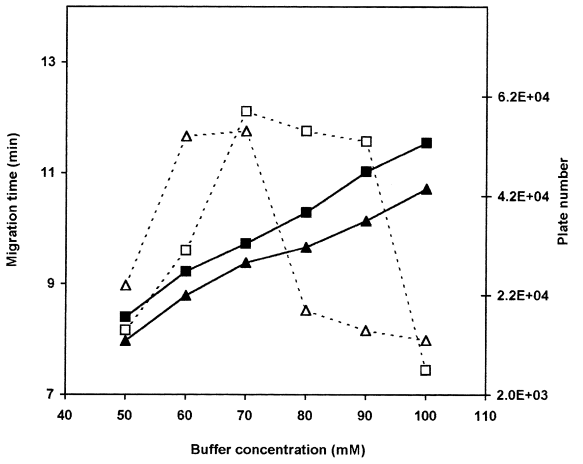

Fig. 3. Influence of concentration of buffer solution. pH 8.6; 13 kV; 25 °C. Migration time: (▲) CLINA, (■) GREPA. Plate number: (△) CLINA, (□) GREPA.

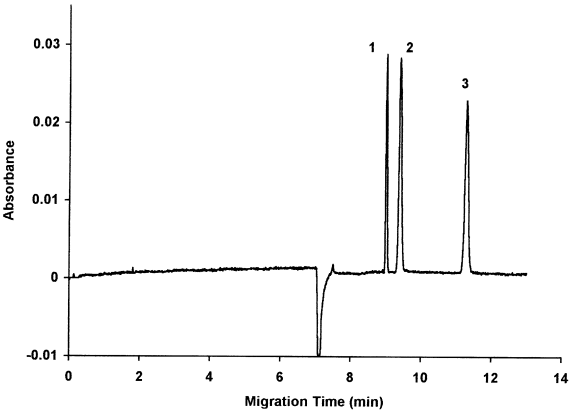

Fig. 5. Typical electropherogram of a standard solution, obtained at the selected optimum conditions. 35 m*M* borate–35 m*M* phosphate (pH 8.6)–acetonitrile (94:6, v/v) as electrophoretic media; 15 kV; 30 °C; injection at 0.5 p.s.i. for 10 s. (1) Clinafloxacin, (2) grepafloxacin, (3) piromidic acid.

trile concentrations (between 0 and 12%, v/v) were tested to study the effect of acetonitrile concentration on the zone spread. The best peak shape was achieved with 6% (v/v).

Voltages from 11 to 19 kV were investigated. The best compromise in terms of resolution, current generated and run time of separation was found to be 15 kV.

The effect of temperature was explored in the range 15–35 °C finding that by increasing the temperature, migration times were reduced. Moreover, above 35 °C the resolution decreased (Fig. 4). Separations were carried out at 30 °C, because migration times were short and resolutions were good.

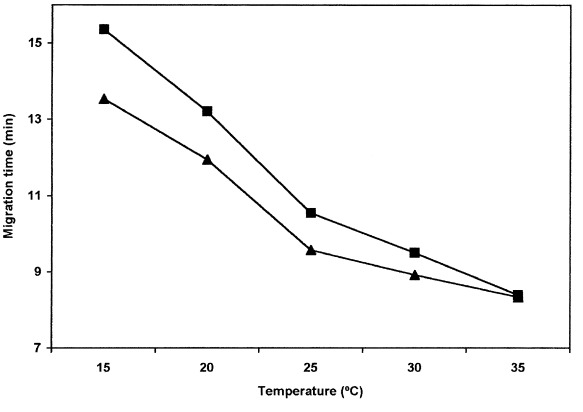

Fig. 4. Influence of temperature. Buffer solution, 94% (35 m*M* borate–35 m*M* phosphate, pH 8.6)–6% acetonitrile (v/v); 15 kV. (▲) CLINA, (■) GREPA.

Fig. 5 shows a representative electropherogram of a standard solution of GREPA, CLINA (20.0 μg ml^{-1} of each analyte) and the internal standard piromidic acid (8.0 μg ml^{-1}) obtained under the optimised conditions. It shows that the electrophoretic procedure selected is adequate for the separation of the analytes involved. The time for one analysis was ~12 min. This time is similar to that reported for GREPA and CLINA as determined by HPLC [8,11].

3.2. Analytical parameters

The calibration graphs are linear for the concentration range 1.0–120.0 μg ml^{-1}. The lack-of-fit test [15] was used to check their linearity. The test is performed by comparing the variability of the current model residuals to the variability between observations at replicate values of the independent variable *x*. Three replicates were used for each of seven prepared standards to obtain each calibration graph. The concentration range of 1.0–120.0 μg ml^{-1} was chosen to cover the expected concentrations in urine after administration of GREPA and CLINA. Thus, Child et al. [16] reported a urinary concentration of 15 μg ml^{-1} of GREPA after a single 400-mg dose (12–24-h interval), while Bron et al. reported that

~50% of the CLINA administered dose was excreted unchanged in the urine with a maximum concentration of 46.7 μg ml^{-1} after a 100-mg dose [17].

The detection limit (LOD) was calculated by comparing the signal-to-noise ratio (S/N) of the lowest detectable concentration to a S/N of 3. The detection limits found were 0.2 and 0.3 μg ml^{-1} for GREPA and CLINA, respectively. A S/N of 10 was applied for the calculation of the quantification limit. The LODs obtained are in accordance with other methods developed to analyze quinolones with a capillary electrophoresis system using UV–Vis detection [18], but better LODs are possible for GREPA and CLINA when analysed by HPLC [8,10]. In CZE the sensitivity of the UV–Vis detector decreases due to the fact that the optical pathlength is restricted to the internal diameter of the capillary.

The precision was measured for both analytes in concentrations of 2, 20 and 70 μg ml^{-1} by performing six independent determinations. CZE analysis yielded good reproducibility (RSD between 1.74 and 3.37%). The analytical parameters are summarised in Table 1.

3.3. Application and validation of the method

3.3.1. Spiked samples

One of the advantages of CZE in routine analysis is the simplicity of sample treatment. In our experiment, we just centrifuged, filtrated and diluted human urine samples spiked with GREPA and CLINA which we then analysed with the procedure described above. Quantifications of GREPA and CLINA concentrations were completed by the standard additions method because there was a matrix effect and the response of analytes decreases in urine samples with respect to standard solutions. Peak identification was made by comparing the migration times and absorption spectra between the peaks corresponding to a standard mixture of GREPA, CLINA and analysed urine samples.

Table 1
Analytical parameters

	Grepafloxacin	Clinafloxacin
Intercept (a)	−0.011	−0.005
Slope (b)	0.0342	0.0178
Correlation coefficient	0.9996	0.9998
Lack-of-fit test (P-value)	0.22	0.42
Linear dynamic range (μg ml^{-1})	1.0–120.0	1.0–120.0
Linearity [1−RSD(b)] (%)	99.6	99.4
Detection limit (μg ml^{-1})	0.2	0.3
Quantification limit (μg ml^{-1})	0.7	1.0
Reproducibility (RSD,%), n=6		
2.0 μg ml^{-1}	2.97	3.37
20.0 μg ml^{-1}	2.11	1.82
70.0 μg ml^{-1}	1.89	1.74

An example of the separation of GREPA and CLINA in human urine is shown in Fig. 6. Other peaks of drugs commonly co-administered with these fluoroquinolones (theophylline, naproxen and acetaminophen) did not interfere with the migration times of the drugs monitored.

The human urine samples were spiked at different levels: 2, 5, 10, 25, 50 and 100 μg ml^{-1}. Validation of the proposed method for these samples was tested by using a recovery test (Student's t-test) [19,20]. As the P-values calculated in all cases are greater than 0.05, the null hypothesis appears to be valid, i.e. recoveries are close to 100% (Table 2).

Validation of the CZE method was also made by comparison with HPLC as a method of reference. Spiked human urine samples with concentrations of

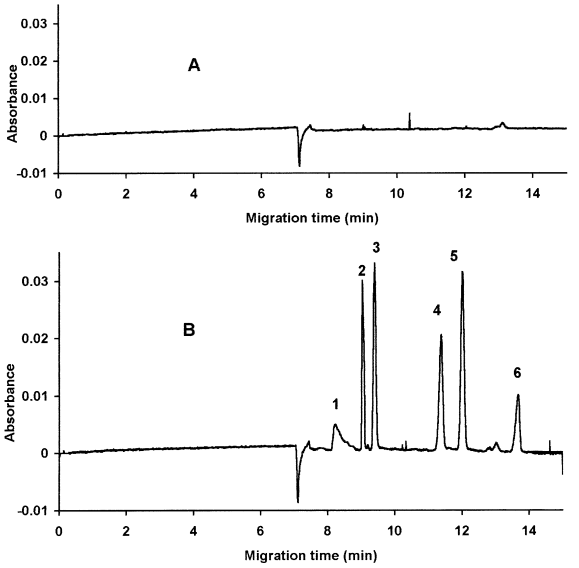

Fig. 6. Electropherogram of: (A) blank human urine sample; (B) spiked human urine sample. All conditions as in Fig. 5. (1) Acetaminophen, (2) clinafloxacin, (3) grepafloxacin, (4) piromidic acid, (5) theophylline, (6) naproxen.

Table 2
Results of recovery assays to check the accuracy of the proposed method for human urine samples

Compound	Sample	Spiked ($\mu g\ ml^{-1}$)	Found[a] ($\mu g\ ml^{-1}$)	Recovery (%)	t	P[b]
Grepafloxacin	Urine-1	2.00	1.92±0.14	96.0	1.48	0.189
		5.00	4.89±0.27	97.8	1.11	0.310
		10.0	10.2±0.5	102.0	0.98	0.364
	Urine-2	2.00	1.93±0.11	96.5	1.67	0.145
		5.00	5.02±0.18	100.4	0.33	0.750
		10.0	9.86±0.35	98.6	1.01	0.351
	Urine-3	25.0	24.3±1.9	97.3	0.90	0.401
		50.0	50.0±2.2	100.1	0.05	0.960
		100.0	98.5±3.6	98.5	1.08	0.320
	Urine-4	25.0	25.0±1.2	100.0	0.09	0.934
		50.0	49.1±4.9	98.2	0.52	0.624
		100.0	99.9±4.9	99.9	0.05	0.958
Clinafloxacin	Urine-1	2.00	1.89±0.16	94.5	1.73	0.133
		5.00	4.89±0.16	97.8	1.72	0.135
		10.0	10.3±0.6	103.0	1.14	0.299
	Urine-2	2.00	1.93±0.14	96.5	1.31	0.238
		5.00	4.96±0.14	99.2	0.79	0.460
		10.0	9.89±0.18	98.9	1.60	0.162
	Urine-3	25.0	25.4±2.5	101.6	0.39	0.712
		50.0	49.2±1.7	98.4	1.26	0.256
		100.0	98.7±2.5	98.7	1.40	0.210
	Urine-4	25.0	24.1±2.1	96.4	1.16	0.288
		50.0	49.7±3.0	99.4	0.27	0.799
		100.0	98.0±5.9	98.0	0.92	0.396

[a] Average value±SD of seven determinations.
[b] P-value of the one-sample comparison test.

10, 30 and 60 $\mu g\ ml^{-1}$ of GREPA and CLINA were analysed using both methods.

The chromatographic separation of grepafloxacin, clinafloxacin and oxolinic acid (internal standard) was achieved at 25 °C using a gradient system. The mobile phase consisted of methanol (eluent A) and a mixture of 93.3% H_3PO_4/NaH_2PO_4 0.02 *M* (pH 2.56) and 6.7% tetrabutyl ammonium bromide 0.1 *M* (eluent B). The chromatography was carried out using the following gradient program: 0–1 min,

Table 3
Comparison of human urine GREPA and CLINA concentrations determined by CZE procedure and HPLC procedure

Compound	Spiked ($\mu g\ ml^{-1}$)	Found[a] ($\mu g\ ml^{-1}$)		t	P[b]
		CZE method	HPLC method		
Grepafloxacin	10.0	10.5±0.3	10.6±0.8	0.4092	0.697
	30.0	29.2±0.6	29.1±1.1	0.2534	0.808
	60.0	57.8±2.3	58.0±0.8	0.2242	0.830
Clinafloxacin	10.0	10.5±0.4	10.5±0.7	0.0875	0.933
	30.0	28.7±2.1	28.9±0.6	0.1820	0.862
	60.0	56.3±1.4	56.8±0.8	0.5723	0.588

[a] Average value±SD of four determinations.
[b] P-value of the two-sample comparison test.

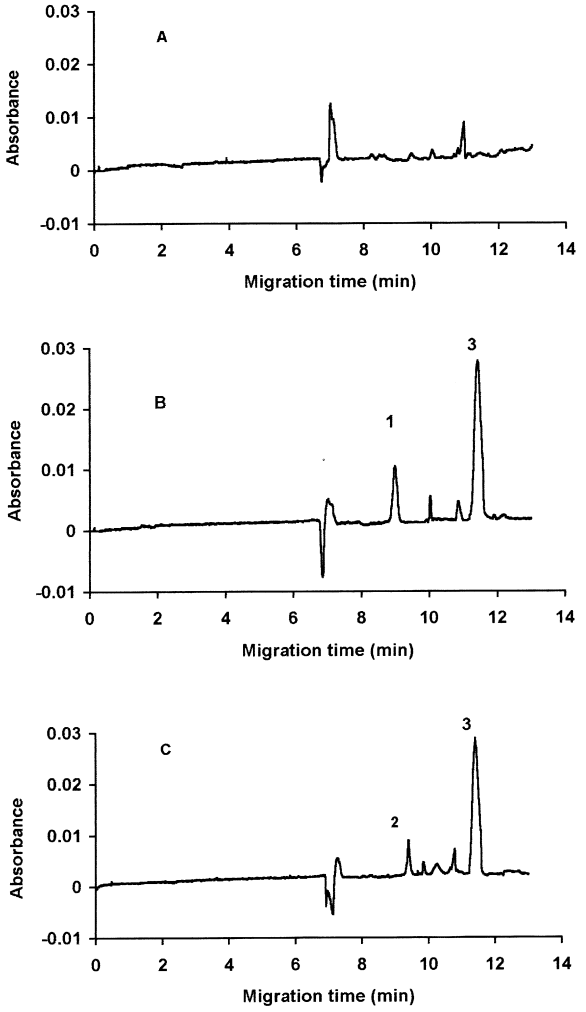

Fig. 7. Electropherogram of: (A) blank rat urine sample; (B) rat urine sample 120 min after administration of 28 mg kg^{-1} of grepafloxacin; (C) rat urine sample 120 min after administration of 13 mg kg^{-1} of clinafloxacin. All conditions as in Fig. 5. (1) Clinafloxacin, (2) grepafloxacin, (3) piromidic acid (internal standard, 20 μg ml^{-1}).

eluent A: 25%, eluent B: 75%; 1–4 min, eluent A: increasing linearly from 25 to 40%; 4–5 min, eluent A: 40%; eluent B: 60%; 5–8 min, eluent A: increasing linearly from 40 to 55%; 8–12 min, eluent A: 55%; eluent B: 45%, after which it was brought back to the initial composition within 5 min. The flow-rate was 0.7 ml min^{-1} and injection volume 20 μl.

The results obtained, summarised in Table 3, show that both methods (CZE and HPLC) yield values within the same range when tested using adequate statistical procedures [21].

3.3.2. Real samples

The proposed method was applied to the determination of GREPA and CLINA in urine samples from a rat (body weight, 245 g) who received a single oral dose of 28 mg kg^{-1} of GREPA [4] and 13 mg kg^{-1} of CLINA. Fig. 7 shows electropherograms of a urine sample, treated as described in Section 2.4, before and 120 min after administration of an oral dose of each drug. The concentrations found (average value±SD of four determinations) were 13.2±0.7 μg ml^{-1} of GREPA and 24.3±1.3 μg ml^{-1} of CLINA.

These samples were also analysed using the HPLC method. Concentrations found were 13.0±0.4 μg ml^{-1} of GREPA and 24.6±0.9 μg ml^{-1} of CLINA.

4. Conclusions

A capillary zone electrophoretic (CZE) method for the determination of bactericide fluoroquinolones grepafloxacin and clinafloxacin in urine samples is presented. The CZE technique is easy to handle and shows good reproducibility. Low amounts of required sample, simple previous sample treatment, good recovery rates and relative short analysis times are the main advantages of this method. It can be useful for clinical and medical researchers.

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