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Simultaneous determination of cefepime and grepafloxacin in human urine by high-performance liquid chromatography

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Abstract

A liquid chromatographic method with UV detection for simultaneous determination of cefepime and grepafloxacin has been developed. The method uses a C_{18} column, equipped with a pre-column of the same material, and acetonitrile–0.1 M phosphoric acid/sodium hydroxide buffer (pH 3.0)–0.01 M *n*-octylamine (pH 3.0) as mobile phase in gradient mode. Mobile flow rate and sample volume injected were 1.3 mL min^{-1} and 20 μL, respectively. Detection wavelengths were 259 nm for cefepime and 278 nm for grepafloxacin. The retention times were 4.03 min for cefepime and 8.85 min for grepafloxacin, with detection limits of 1.0 and 1.1 μg mL^{-1}, respectively. The method was applied to the determination of both antibiotics in spiked samples of human urine.

Keywords: Cefepime; Grepafloxacin; HPLC; Human urine

1. Introduction

Two-drug combinations have been used to treat infections caused by non-fermentative Gram-negative bacteria (*Acenetobacter baumannii*, *Pseudomonas aeruginosa* and others), as they are less susceptible to many antimicrobial agents and are becoming increasingly resistant. The combination of a β-lactam and a fluoroquinolone has shown better results than those found for the traditional β-lactam and aminoglycoside combination, with a decrease in resistance development [1–3].

The activity of the β-lactam cefepime in combination with fluoroquinolones has been studied in the literature, and synergy has been detected against some drug-resistant pathogens [2,4–6], but the analytical literature about the topic is very scarce. The present work proposes a sensitive, rapid and simple HPLC method for the simultaneous determination of cefepime and grepafloxacin. The method has been applied to the determination of both antibiotics in human urine.

Cefepime, [2-aminothiazol-4-yl]-2(*Z*)-[methoxy-iminoacetamido]-3-[methyl-1-pyrrolidino]methyl-ceph3-em4-carboxylic acid is a new injectable fourth-generation β-lactam cephalosporin with a positively charged quaternised *N*-methyl-pyrrolidine substitution at the 3 position of the cephem nucleus and a $pK_a = 2.7$ [7]. Cefepime is administered in 2, 1 or 0.5 g per 8-h doses to patients with pneumonia, uncomplicated or complicated urinary tractor or skin infections and complicated intra-abdominal infections.

Grepafloxacin (1-cycloproplyl-6-fluoro-1,4-dihydro-5-methyl-7-(3-methyl-1-piperazinyl)-4-oxo-3-quinolinecarboxylic acid) is a synthetic fluorinated quinolone derivative with a $pK_a = 6.32$ [8], administered to patients with urinary, respiratory or cutaneous infections in 400 mg d^{-1} doses. Both antibiotics have a broad spectrum of activity against many Gram-positive and Gram-negative bacteria. Cefepime and grepafloxacin are mainly excreted in urine in unaltered form, with typical final concentrations over the ranges 10–200 μg mL^{-1} and 4–80 μg mL^{-1}, respectively.

Several methods have been reported for the determination of cefepime and grepafloxacin. Second derivative spectroscopy [9] and polarographic techniques [10,11] have been used for the quantitation of cefepime, while capillary-zone

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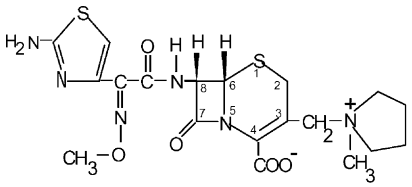
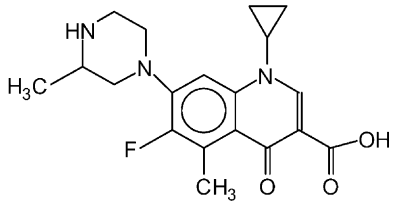
electrophoresis [12] and luminescence techniques [13,14] has been applied to the determination of grepafloxacin.

With respect to chromatographic techniques, HPLC with UV detection has been applied to stability [15,16] and pharmacokinetic [17] studies for cefepime. This technique has also been applied to the determination of this antibiotic in human plasma [18–20] and urine and dialysis fluid [20], with detection wavelengths over the range 255–280 nm. For grepafloxacin, HPLC with UV detection at 280 nm has been applied to the determination of this fluoroquinolone in urine and serum [21,22], and fluorescence detection (with excitation at 338 nm and emission at 425 nm) has been applied for plasma samples [23]. No method for the simultaneous determination of these antibiotics has been found in the literature.



Cefepime



Grepafloxacin

2. Experimental

2.1. Chemicals

Cefepime and grepafloxacin were kindly provided by Bristol-Myers-Squibb (Madrid, Spain) and Glaxo SmithKline (London, UK), respectively. Methanol and acetonitrile of HPLC grade, phosphoric acid, sodium hydroxide and octylamine were provided by Merck. De-mineralised water obtained from a Milli-Q treatment system (Millipore, Milford, MA, USA) was used throughout. Urine was obtained from healthy volunteers and kept at −20 °C in sterile containers.

Aqueous stock solutions (1 mg mL^{-1} cefepime and 0.5 mg mL^{-1} grepafloxacin) were prepared weekly and stored in the dark at 4 °C. Under these conditions the stock solution remained stable at least 2 weeks. Working solutions with 100 μg mL^{-1} cefepime and 50 μg mL^{-1} grepafloxacin were prepared daily by diluting this stock solution with water. No internal standard was needed, as both antibiotics were quantified by external calibration method.

Table 1
Optimised gradient

Time (min)	A (%)	B (%)	C (%)
0	8	46	46
4	8	46	46
6	36	32	32
10	36	32	32
12	8	46	46

(A) Acetonitrile, (B) 0.1 M phosphoric acid/sodium hydroxide buffer (pH 3.0) and (C) 0.01 M *n*-octylamine (pH 3.0).

2.2. Chromatographic conditions

The liquid chromatographic system (Merck-Hitachi-Lachrom, Barcelona; Spain) consisted of a pump (Model L-7100) connected via a reversed-phase LiChrospher 100 RP-18 column (5 μm, 250 × 4 mm i.d. LichroCART) to a UV–vis detector diode-array (Merck-Hitachi, Model L-7455). In order to avoid the introduction in the column of molecules that could interact with C18 in non-reversible ways, a pre-column (10 × 4 mm i.d.) packed with the same packing material was fitted just before the inlet junction of the analytical column. The injector was a Rheodyne (Model 7725i) manual injection valve, fitted with a 20 μL sample loop. Chromatograms were processed by a HPLC-System-Manager HSM D-7000 (Merck-Hitachi).

Mobile phase used was composed of three solvents: acetonitrile (A), 0.1 M phosphoric acid/sodium hydroxide buffer with pH 3.0 (B) and 0.01 M *n*-octylamine solution with pH 3.0 adjusted with phosphoric acid (C). A flow rate of 1.3 mL was employed, applying a gradient resumed in Table 1. Detection was carried out measuring the absorbance at the wavelengths-program resumed in Table 2. After the measurements, the HPLC system was cleaned with de-mineralised water (30 min, with a 0.5 mL flow rate) and methanol (45 min, with 0.5 mL flow rate).

2.3. Procedures

2.3.1. Calibration graphs

A series of 10 standard solutions (three replicates for each one) of cefepime and grepafloxacin were prepared with concentrations over the ranges 10–200 and 4–80 μg mL^{-1}, respectively. A 20 μL volume from each solution was injected in the chromatographic system, in the conditions detailed previously, and their respective calibration graphs of peak

Table 2
Detection wavelengths

Time (min)	Wavelength (nm)
0–3	350
3–5	259
5–8	350
8–9.5	278
9.5–10	350

area (Y) versus antibiotic concentration (X) were obtained by application of the least-square method to the obtained results.

2.3.2. Determination of cefepime and grepafloxacin in human urine

Stocks of human drug-free urine from healthy volunteers were spiked with different amounts of cefepime and grepafloxacin. Samples of 10 mL urine were spiked for final concentrations of 10–200 $\mu g\,mL^{-1}$ and 4–80 $\mu g\,mL^{-1}$, respectively. The spiked samples were microfiltered through a 0.45 μm filter. A 20 μL volume of each sample was injected into the system, and their respective chromatograms were obtained in the experimental conditions resumed previously.

3. Results and discussion

3.1. Preliminary studies. Influence of the composition and flow rate of the mobile phase

The preliminary studies were carried by the injection of a mixture of cefepime and grepafloxacin 20 and 8 $\mu g\,mL^{-1}$, respectively, in the HPLC system. Mobile phase consisted in three different solvents: acetonitrile (A), phosphoric acid/sodium hydroxide buffer (B) and *n*-octylamine solution (C) as ion-pairing agent. For these preliminary experiences, a 0.1 M buffer concentration (pH 3.0) and a 0.01 M *n*-octylamine (pH 3.0) concentration were employed. Flow rate of mobile phase was 1.0 mL^{-1}.

The preliminary experiences shown that a well-defined peak was achieved for cefepime with 8–15% acetonitrile in the mobile phase. Successive chromatographic scans with higher acetonitrile concentrations led to an overlap between cefepime peak and t_0 (2.2 min). On the other hand, with lower than 15% actonitrile, the retention time for grepafloxacin was higher than 25 min. In order to obtain better retention times, it was decided to apply a gradient of elution, starting with a low acetonitrile percentage and increasing it as the separation went by. Different gradients were tested: Fig. 1 shows the chromatogram obtained with an 10% acetonitrile in a first stage (0–5 min), followed with an increase to in acetonitrile percentage (5–8 min) until reaching a constant 25% value (8–20 min). The best results in terms of resolution and run time were obtained with the gradient resumed in Table 1 (with a 8% acetonitrile in a first stage that led to the elution of cefepime and a 36% acetonitrile in a seconde stage that led to the elution of grepafloxacin), so this gradient profile was selected and applied in further studies.

Simultaneously, the influence of the flow rate was also studied over the range 0.5–1.5 mL^{-1}, modifying accordingly the time program for the gradients tested for each rate. It was shown that the best results were achieved when a 1.3 mL^{-1} flow rate was selected in the elution conditions detailed previously, as lower rates led to worse-defined peaks (with a decrease of 30–40% in analytical signals for both antibiotics at the lower rate tested), and higher rates led to worse resolutions and reproducibility, so this value was selected for the proposed procedure.

3.2. Selection of detection wavelength

In order to achieve more sensitive results, the detection wavelength was modified as the separation went by as shown in Table 2. These selected wavelengths corresponded to the maximum absorption values for cefepime (259 nm) and grepafloxacin (278 nm) while each molecule was being detected, and to the minimum absorption value for the mobile phase (350 nm) before and after the apparition of both peaks.

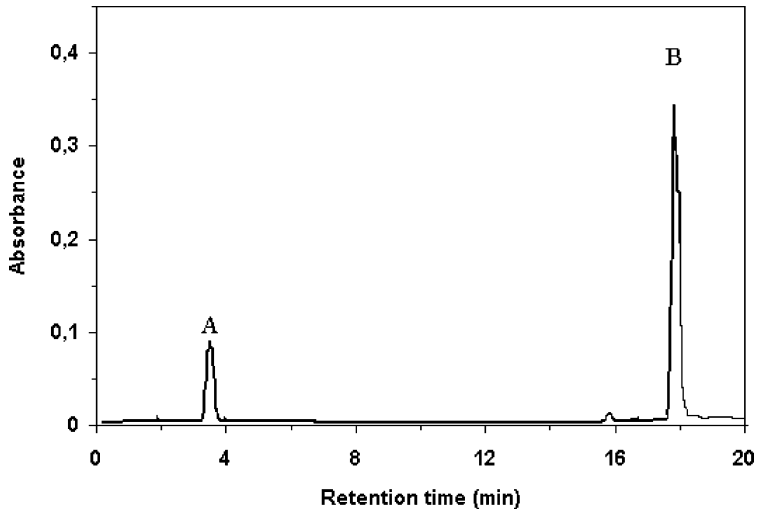


Fig. 1. Chromatogram of a standard solution 50 $\mu g\,mL^{-1}$ cefepime (A) and 20 $\mu g\,mL^{-1}$ grepafloxacin (B). Acetonitrile in mobile phase: 10% (0–5 min) and 25% (8–20 min).

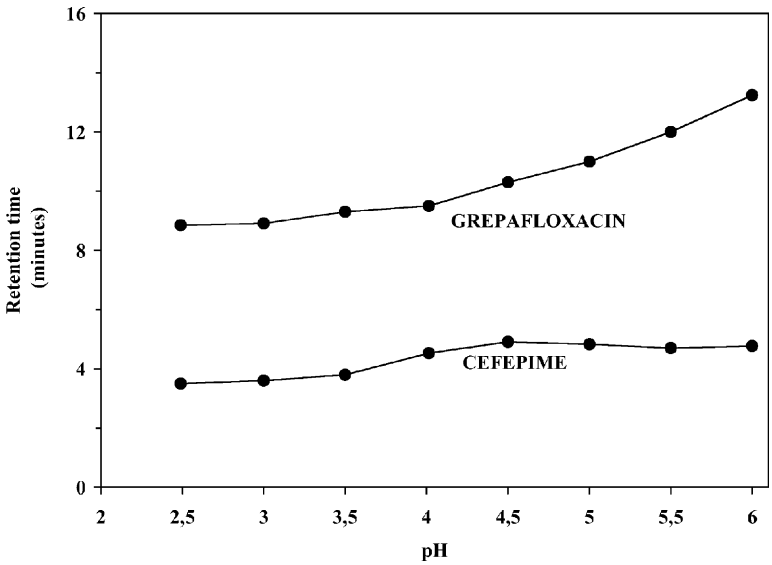


Fig. 2. Influence of the pH in the retention times of cefepime and grepafloxacin when separation with an acetonitrile–0.1 M phosphoric acid/sodium hydroxide–0.01 M *n*-octylamine as mobile phase.

3.3. Influence of the pH

The influence of the pH of the solutions B and C in the retention times of cefepime and grepafloxacin was studied for pH values over the range 2.5–6.0, with 0.1 M phosphate buffer and 0.02 M *n*-octylamine concentrations, respectively. As can be seen in Fig. 2, retention time for cefepime showed a small increase with the pH, while the retention time for grepafloxacin increased notably for higher pH values (from 8.81 min at pH 3.0 to more than 13 min at pH 6.0). Nevertheless, retention time for both cefepime grepafloxacin kept a constant value over the pH range of 2.5–3.5. Thus, a pH of 3.0 was selected as optimum for further studies.

3.4. Influence of the buffer concentration

The influence of the buffer concentration of the mobile phase was studied by changing it from 0.1 to 0.8 M in the solution B. The retention times for grepafloxacin kept unaffected, but a small increase for the retention time of cefepime was observed for higher than 0.4 M buffer concentrations, from 4.03 min with 0.1–0.4 M buffer (Fig. 3) to 5.12 min with 0.8 M (Fig. 4). Thus, a 0.1 M buffer concentration was selected as suitable for the proposed method, as it kept the mobile phase pH properly and led to the lowest absorption.

3.5. Influence of n-octylamine concentration

n-Octylamine was employed as ion-pairing agent, as preliminary studies in its absence of this agent led to an overlap between grepafloxacin peak and t_0. The concentration of *n*-octylamine in the solution C was optimised by study the retention times for cefepime and grepafloxacin obtained with concentration of *n*-octylamine over the range 0.005–0.05 M, in the previously optimised working conditions. Retention

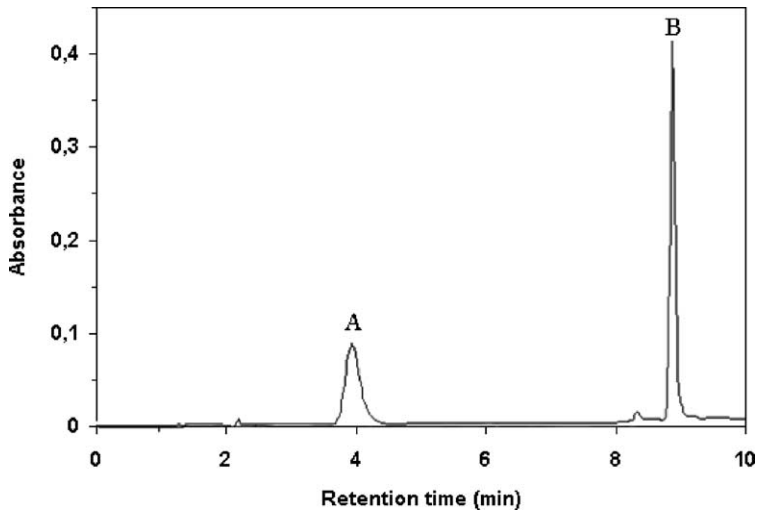


Fig. 3. Chromatogram of a standard solution 50 μg mL^{-1} cefepime (A) and 20 μg mL^{-1} grepafloxacin (B) with 0.1 M buffer concentration.

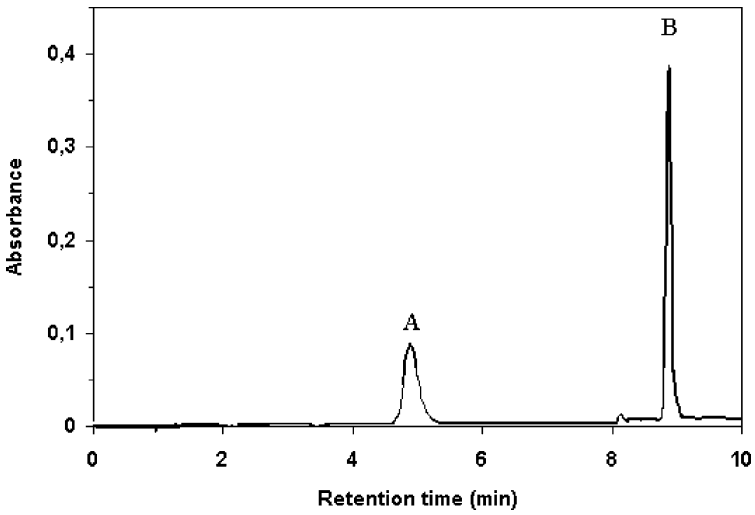


Fig. 4. Chromatogram of a standard solution 50 μg mL^{-1} cefepime (A) and 20 μg mL^{-1} grepafloxacin (B) with 0.8 M buffer concentration.

times for both grepafloxacin and cefepime kept unchanged, so a 0.01 M concentration was selected for the optimised procedure.

3.6. Figures of merit of the determination of cefepime and grepafloxacin

3.6.1. Robustness

Once all variables were optimised, the influence of some experimental parameters on the results was tested. As reference, Fig. 3 shows the chromatogram obtained for a 50 μg mL^{-1} cefepime and 20 μg mL^{-1} grepafloxacin sample following the proposed method. The retention times, in these conditions, were 4.03 min for cefepime and 8.85 min for grepafloxacin.

It was found that variations in mobile phase pH of ±0.75 with respect to the optimum value of pH 3.0 led to lower than 5% variations in the analytical signals (peak area) and retention times for both cefepime and grepafloxacin. Variations found for phosphate buffer concentration over the range 0.1–0.4 M were also lower than 5% for both retention times and peak areas. No significant variation was found for *n*-octylamine concentration over the range 0.005–0.05 M.

With respect to mobile phase composition, no significant variations were found when acetonitrile was kept in a 6–9% range in the first stage of the gradient (0–4 min). Below or above this range, higher than 5% variations were found in retention time and area peak for cefepime, but no significant variations were found to grepafloxacin. For the second stage (6–10 min), variations in retention time and peak area for grepafloxacin were lower than 5% for acetonitrile over the range 32–40%.

3.6.2. Calibration results

A series of standard solutions with increasing cefepime and grepafloxacin concentration, were injected in the HPLC system, as explained in Section 2.3. The calibration graphs were obtained plotting the peak area for each compound versus their respective concentration. It was found a linear relationship between concentration and peak area over the ranges for 10–200 μg mL^{-1} cefepime and 4–80 μg mL^{-1} for grepafloxacin. Table 3 resume the regression parameters obtained for each compound by the application of the least-square method.

3.6.3. Accuracy and precision

Accuracy and intra-run precision were calculated measuring 11 replicates of target solutions 150, 100 and 50 μg mL^{-1} cefepime and 60, 40 and 20 μg mL^{-1} grepafloxacin. Inter-run study was carried out measuring 11 replicates of the same solutions over a 10-day period. The results obtained are shown in Table 4. In order to determine if the obtained values were statistically different than the real values, a Student's *t*-test was performed [24]. Table 4 resumes the $|t|$ values for each concentration: as can be seen, all those values were lower than the critical tabulated $|t|$ (2.23, $P = 5\%$). Thus, there was no difference, statistically, between the real and the obtained concentrations.

Table 3
Analytical characteristics of the determination of cefepime and grepafloxacin

Parameter	Cefepime	Grepafloxacin
a	0.28	0.18
S_a	0.08	0.02
b	0.1586	0.609
S_b	0.0007	0.004
r	0.9998	0.9992
R.S.D. (%)	3.1	1.0
DL (μg mL^{-1})	1.0	1.1
QL (μg mL^{-1})	3.3	3.5

a, intercept; *b*, slope; S_a, intercept standard deviation; S_b, slope standard deviation; *r*, correlation coefficient; R.S.D., relative standard deviation (100 μg mL^{-1} cefepime and 40 μg mL^{-1} grepafloxacin); DL, detection limit; QL, quantification limit.

Table 4
Accuracy and precision study for cefepime and grepafloxacin determination

Cefepime concentration ($\mu g\,mL^{-1}$)	Grepafloxacin concentration ($\mu g\,mL^{-1}$)	Mean ± S.D. ($\mu g\,mL^{-1}$)	$\lvert t\rvert$	R.S.D. (%)	Mean ± S.D. ($\mu g\,mL^{-1}$)	$\lvert t\rvert$	R.S.D. (%)
Intra-run study							
150	60	145.7 ± 4.9	0.19	3.4	59.6 ± 0.7	1.81	1.2
100	40	99.4 ± 3.1	0.61	3.1	40.2 ± 0.4	1.05	1.0
50	20	50.3 ± 1.8	0.53	3.6	19.8 ± 0.3	2.1	1.6
Inter-run study							
150	60	151.6 ± 5.5	0.92	3.6	59.3 ± 1.9	1.17	3.2
100	40	100.8 ± 3.4	0.74	3.4	40.4 ± 0.8	1.58	2.0
50	20	49.5 ± 2.0	0.79	4.0	19.8 ± 0.5	1.26	2.5

3.6.4. Detection and quantitation limits

Detection and quantitation limits were calculated as 3 and 10 times the standard deviation, respectively, obtained measuring 11 replicates of a solution with cefepime and grepafloxacin concentrations corresponding to the lowest values of their calibration ranges (8.0 and 4.0 $\mu g\,mL^{-1}$, respectively), as they were the lowest ones which could be used. The detection limits achieved for cefepime and grepafloxacin were 1.0 and 1.1 $\mu g\,mL^{-1}$, while the quantitation limits were 3.3 and 3.5 $\mu g\,mL^{-1}$, respectively.

3.6.5. Determination of cefepime and grepafloxacin in urine

Fig. 5 shows (1) the chromatogram obtained for blank urine in the optimised experimental conditions and (2) the chromatogram obtained for urine spiked with 100 $\mu g\,mL^{-1}$

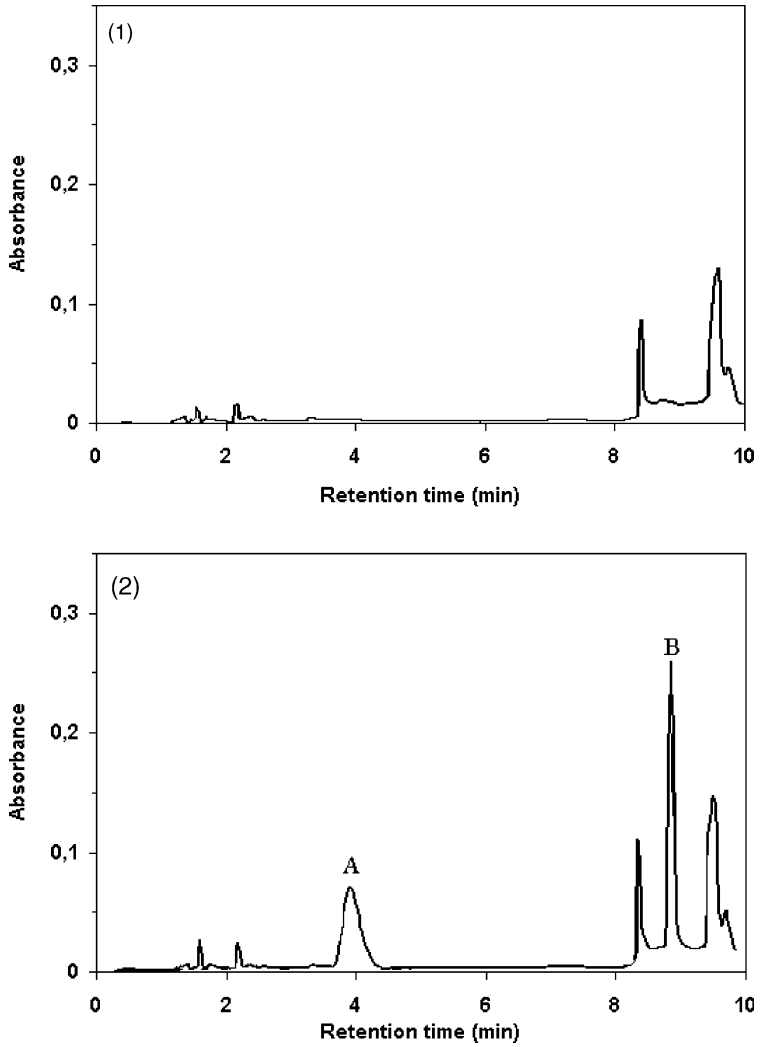


Fig. 5. Chromatograms of (1) a blank urine sample and (2) a urine sample spiked with 100 mg mL^{-1} cefepime (A) and 40 mg mL^{-1} grepafloxacin (B).

Table 5
Results of the cefepime and grepafloxacin determination in urine

Cefepime concentration	Recovery ± S.D.(%)	$\|t\|$	Grepafloxacin concentration	Recovery ± S.D.(%)	$\|t\|$
Intra-run study					
10	103.8 ± 8.6	0.98	4	100.5 ± 9.7	0.12
50	96.7 ± 3.7	1.99	20	94.7 ± 4.7	2.52
100	95.8 ± 3.9	2.41	40	99.3 ± 2.8	0.56
200	101.4 ± 2.0	1.57	80	97.1 ± 2.6	2.49
Inter-run study					
10	104.2 ± 9.5	0.99	4	101.5 ± 10.2	0.31
50	98.1 ± 5.2	0.82	20	94.3 ± 5.7	2.24
100	94.7 ± 4.5	2.63	40	98.2 ± 3.5	1.15
200	101.8 ± 2.8	1.44	80	97.5 ± 3.1	1.80

cefepime (A) and 40 $\mu g\,mL^{-1}$ grepafloxacin (B). Calculated resolution (R_S) was found to be 2.31 for cefepime and 2.04 for grepafloxacin. As a $R_S \geq 1.5$–2.0 is generally accepted as a good resolution between the peak and the closest electing potential interference, these results show that the peaks for both antibiotics were resolved from the other components of this biological fluid.

The optimised method was applied to the determination of cefepime and grepafloxacin in a series of urine samples (spiked at different antibiotic concentrations) as described in Section 2.3. Table 5 resumes the recoveries obtained for each spiked concentration (with five replicates for each concentration) within an intra-run assay. Again, in order to determine if the experimental recoveries were statistically different than 100%, a Student's *t*-test was performed. All calculated $|t|$ values for each concentration (Table 5) were lower than the critical tabulated $|t|$ (2.78, $P = 5\%$), so there was no difference, statistically, between the spiked and the obtained concentrations.

Lastly, a series of analysis of urine samples spiked with the same concentrations and number of replicates as the intra-run assay was carried out over a range of 10 days. The recoveries obtained are shown in Table 5. Again, obtained recoveries were no statistically different than 100%, as all calculated $|t|$ values were lower than the critical tabulated $|t|$ (2.78, $P = 5\%$).

4. Conclusions

A fast, easy and simple chromatographic procedure has been developed for the separation and quantitation of cefepime and grepafloxacin. By the application of a gradient elution, the separation was achieved in 10 min, with detection limits of approximately 1.0 $\mu g\,mL^{-1}$ for both antibiotics. The method has been applied to the simultaneous determination of both antibiotics in human urine.

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