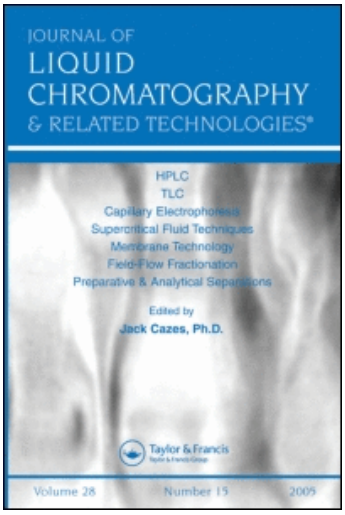

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SIMULTANEOUS SEPARATION AND DETERMINATION OF QUINOLONES IN PHARMACEUTICALS BY MICELLAR LIQUID CHROMATOGRAPHY

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□ *A rapid and simple liquid chromatographic procedure using micellar mobile phases is reported for the separation and determination of four quinolones (pipemidic acid, levofloxacin, norfloxacin, and moxifloxacin) in pharmaceuticals.*

This purpose was achieved without any previous pretreatment step in a C_{18} column using a micellar mobile phase of 0.15 M sodium dodecyl sulphate, 2.5% propanol, and 0.5% triethylamine at pH 3, with retention times below 12 min. For detection, the diode-array UV-Vis set at 276 nm was used. The limits of detection and quantification were between 8 – 51 and 28 – 171 ng/mL, respectively. This method was validated in terms of intra-day and inter-day precision and accuracy, and robustness. Calibration curves over the concentration range of 0.1 – 50 μg/mL were linear ($r^2 > 0.9997$). Good claim percentages (96 – 106%) were obtained in the analysis of pharmaceutical formulations. The results show that the procedure is suitable for the routine analysis of drugs.

Keywords direct injection, micellar mobile phase, pharmaceutical formulation, quinolone, SDS

INTRODUCTION

Quinolones and fluoroquinolones are synthetic antibiotics whose action is based on their anti-DNA activity. Since nalidixic acid was discovered,[1] a number of structure modifications to the quinolone nucleus have been performed to increase antimicrobial activity and to enhance the pharmacokinetic performance of these drugs. The general structure consists of a 1-sustituted-1,4-oxopyridine-3-carboxylic moiety combined with either an aromatic or heteroaromatic ring. Fluoroquinolones are quinolones with a fluorine atom at position 6 of the quinolone naphthyridine or benzoaxazine ring systems, and belong to the second generation of quinolones. They are

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characterised by their greater effectiveness against bacterial activity,[2] and are used in both human and veterinary medicine. In humans, they are used to treat an extensive range of diseases, including urinary, respiratory, and gastrointestinal tract infections.[3]

The analysis of quinolones has traditionally been performed using microbiological methods. However, this technique is time consuming and offers poor precision and specificity. Other non-routine techniques, such as terbium (III)-sensitised luminescence,[4] capillary electrophoresis,[5–7] or immunoaffinity chromatography,[8] have also been applied.

Last generation LC-MS-(MS) equipment has also been used,[9–11] although this equipment is very expensive and only a few laboratories can afford such instrumentation. High performance liquid chromatography (HPLC) has become an important tool for the analysis of single and various combinations of quinolones in biological fluids, foods, environmental samples, and pharmaceutical preparations using either UV or fluorescence as the detection method.[12–27]

Micellar chromatographic (MLC) methods offer the advantages of the direct injection of samples with no pretreatment other than filtration and the low toxicity of the mobile phases employed.[28] MLC has proven to be a

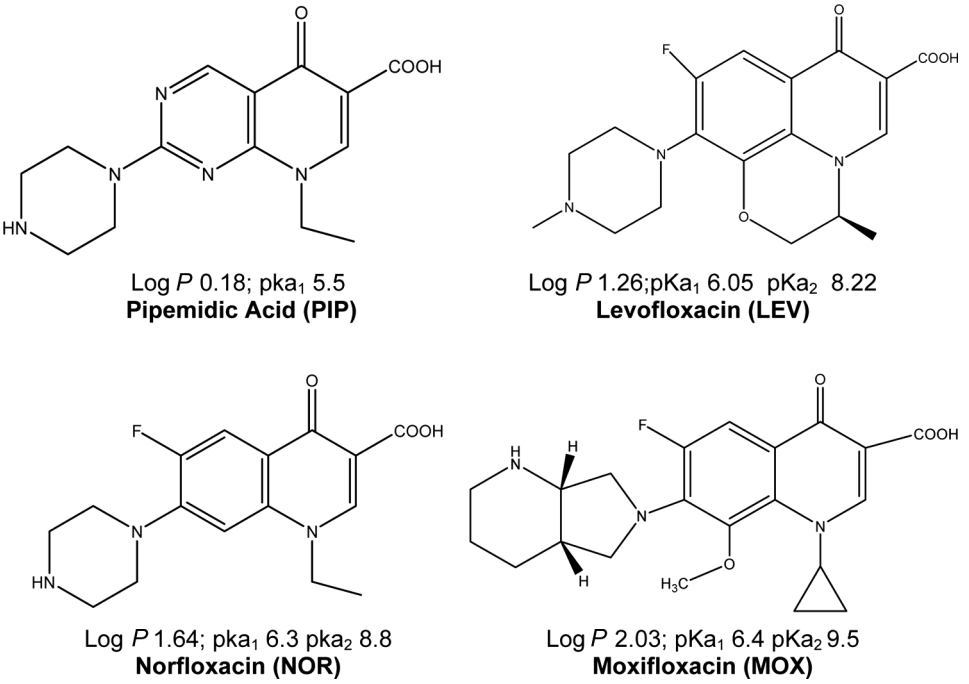

FIGURE 1 Structures, octanol-water partition coefficient (log *P*) and acid-based constants of the studied quinolones.[34,36]

useful technique in the determination of diverse groups of drugs, such as thiazide diuretics,[29,30] furosemide,[31] and trazodone[32] in pharmaceutical formulations.

In this work, a simple chromatographic procedure with micellar mobile phases of Sodium Dodecyl Sulphate (SDS) for the simultaneous determination of pipemidic acid, levofloxacin, norfloxacin, and moxifloxacin (Figure 1) has been developed and applied to control numerous pharmaceuticals in several dosage forms, and validated according to the ICH harmonised tripartite guideline.[33] Although these compounds are not administered together, the proposed method allowed the determination and quantification of the four quinolones in a single chromatographic run without modifications being necessary for each compound separately, which make the proposed method more economic and faster.

EXPERIMENTAL

Reagents and Samples

Pipemidic acid (PIP) and norfloxacin (NOR) were purchased from Sigma (St. Louis, MO, USA), moxifloxacin (MOX) from Bayer (Leverkusen, Germany), and levofloxacin (LEV) from Fluka (Milan, Italy). Distilled deionised water was used throughout. Sodium dodecyl sulphate (SDS), sodium dihydrogen phosphate, and methanol were obtained from Merck (Darmstadt, Germany). Propanol was purchased from Scharlab (Barcelona, Spain). Hydrochloric acid and triethylamine were acquired from J. T. Baker (Deventer, the Netherlands).

Instrumentation

The pH of the solutions was measured with a Crison GLP 22 (Barcelona), equipped with a combined Ag/AgCl/glass electrode. The balance used was a Mettler-Toledo A×105 Delta-Range (Greifensee, Switzerland). The vortex shaker and sonification unit were acquired from Selecta (Barcelona). The chromatographic system was an Agilent Technologies Series 1100 (Palo Alto, CA, USA) equipped with a quaternary pump, an autosampler, and a UV-Visible detector.

Chromatographic Conditions

A reversed phase Kromasil C_{18} column (150 mm × 4.6 mm, 5 μm particle size) (Scharlab) was used. The selected mobile phase was 0.15 M SDS, 2.5% (v/v) propanol and 0.5% triethylamine at pH 3. The flow rate

and injection volume were 1 mL/min and 20 μL, respectively. Experiments were carried out at room temperature and detection was performed at 276 nm. Chromatographic signals were acquired and processed with an Agilent ChemStation (Rev. A.10.01).

Mobile and Standard Solutions Preparation

The micellar mobile phase was prepared using SDS and 0.5% (v/v) of triethylamine, which was buffered with sodium dihydrogen phosphate 0.01 M at pH 3 using HCl 0.1 M and, lastly, propanol was added to obtain the desired concentration.

Stock solutions of 50 μg/mL of each compound were prepared. Drugs were dissolved in ethanol with the help of an ultrasonic bath and topped up to the mark on the volumetric flask with a 0.1 M SDS solution buffered with phosphate at pH 3. For the analysis of the drugs, several standard solutions were prepared in the 1–25 μg/mL range. Fresh solutions were prepared periodically.

Pharmaceutical Sample Preparation

The pharmaceuticals analysed were tablets and coated capsules. The average weight per tablet was calculated from ten units. Tablets were ground and reduced to a fine homogeneous powder in a mortar. Several portions of this powder were accurately weighed and sonicated in the presence of ethanol (5% v/v of the final content) in an ultrasonic bath. Then 0.1 M SDS solution at pH 3 was added to favour the extraction of the analyte, and the ultrasonic bath was used again. The excipients in the tablets were not soluble in the micellar medium. Therefore, sample solutions were filtered before being injected into the chromatograph. However, filtration was always performed directly into the autosampler vials through 0.45 μm nylon membranes (Micron Separations, MA, USA).

RESULTS AND DISCUSSION

Mobile Phase Selection

Preliminary studies were carried out to select an efficient method for the analysis of four quinolones. Parameters, such as detection wavelength, mobile phase composition, percentages, and optimum pH, have been thoroughly studied.

Several mobile phases were investigated using different alcohols and percentages. Propanol yielded better efficiencies but larger retentions than butanol. However, the peaks of the compounds could not be resolved with

butanol. Thus, propanol was preferred to optimise the separation of the four drugs.

Quinolones have two ionisable functional groups: carboxylic acid and N_4 of the piperazine ring. The carboxylic group is normally a stronger acid than the ammonium group and has a pka_1 value ranging from 5.5 to 9.5 in water. The pka values and octanol-water partition coefficients (log *P*) are shown in Figure 1.[34–36] Among the different pH media tested, pH 3 was chosen because good retention times were obtained with narrow and well resolved peaks. Furthermore, efficiencies deteriorated when the pH of the mobile phase was increased.

Bonded silica phases are problematic from the point of view of pH stability and residual chemical activity of the unprotected silica support, which can induce tailing peaks and variable retention times for basic compounds. Using an amine, such as thiethylamine (TEA), is a common practice to protect the silanol groups of the stationary phase in order to increase peak efficiencies for basic compounds with amine groups. The addition of TEA enhanced the efficiencies of the four quinolones. However, this amine behaved as another modifier and the retention factors of the compounds lowered. For these reasons, the TEA concentration was limited to 0.5%.

Optimization Strategy

An optimization study for the mixture of PIP, LEV, NOR, and MOX was done. An adequate control of the concentrations of both the surfactant and modifier can lead to chromatograms presenting a good resolution and sufficient elution strength.

In order to optimize the mobile phase composition, the retention equation of the four quinolones was obtained using a reduced (five) and selected number of mobile phases, four located at the corners of a rectangular factor space and the fifth in its centre. The limits of the factor space (surfactant and alcohol) were in the 0.05 M to 0.15 M and 2.5 to 12.5% ranges for the concentration of SDS and the volume of propanol, respectively. The errors in the retention factors predicted with these equations were below 2% for all the compounds. The retention factors (k), efficiencies (N), and asymmetry factors (B/A) of the four compounds were measured and processed with the Michrom software,[37,38] which helps to model the compounds retention by taking into account the maximum resolution factor and the minimum analysis time. The equation used was:

$$k = \frac{K_{AS}\dfrac{1}{1 + K_{AD}\varphi}}{1 + K_{AM}\dfrac{1 + K_{MD}\varphi}{1 + K_{AD}\varphi}[M]} \quad (1)$$

where [M] and φ are the concentrations of the surfactant and modifier; K_{AS} and K_{AM} correspond to the equilibria between the solute in bulk water and the stationary phase or micelle, respectively; K_{AD} and K_{MD} measure the relative variation in the concentration of the solute in bulk water and micelles due to the presence of a modifier, as compared to a pure micellar solution (without a modifier).

The global resolution diagram, and the simulated and real chromatograms for the optimum mobile phase obtained are depicted in Figure 2. As can be seen in Figure 2a, resolution values close to one (maximum value) can be obtained in a narrow region of SDS (0.12–0.15 M) and propanol (2.5–3.5% (v/v)). The best resolution value was obtained for a composition of 0.15 M SDS–2.5% propanol–0.5% TEA–0.01 M NaH_2PO_4

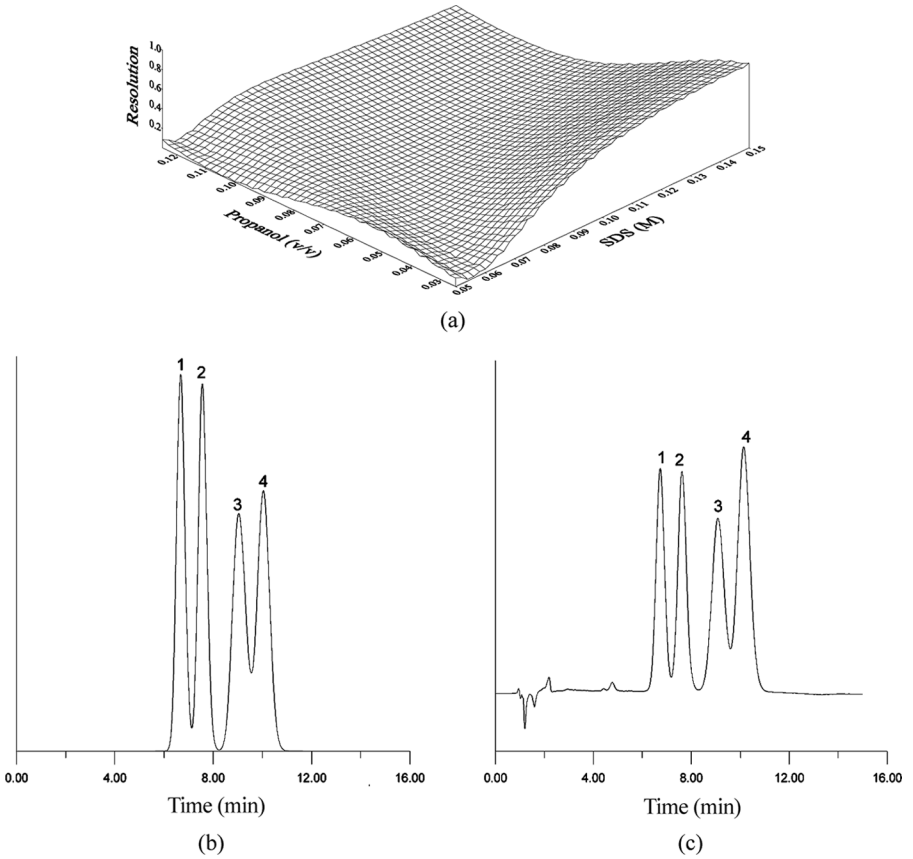

FIGURE 2 (a) Global resolution diagram, (b) simulated, and (c) real chromatogram for a mixture of (1) PIP (1.25 μg/mL), (2) LEV (5 μg/mL), (3) MOX (10 μg/mL), and (4) NOR (2.5 μg/mL). Mobile phase: 0.15 M SDS–2.5% 1-propanol–0.5% thiethylamine–pH 3, flow rate: 1 mL/min, UV detection at 276 nm.

at pH 3 with an analysis time below 12 minutes. Thus, this mobile phase was selected as optimum. Figure 2b and 2c show the simulated and experimental chromatogram for the mixture of the four quinolones in the optimum mobile phase. The agreement between both is excellent. The chromatographic parameters (k, N, and B/A) obtained were: 6.6, 1900, and 1.1; 5.7, 1500, and 1.1; 9.1, 1700, and 1.0; and 8.1, 1100, and 1.2 for PIP, LEV, NOR, and MOX, respectively.

VALIDATION

The ICH harmonised tripartite guideline[33] was followed to validate the method.

Linearity

Under the selected chromatographic conditions, the linear range of the signal response for each drug was studied over the concentration range of $0.1 - 50\,\mu g/mL$. Seven different concentration levels (0.1, 0.5, 1, 5, 15, 25, $50\,\mu g/mL$) were obtained for each standard solution, and were conveniently diluted with 0.1 M SDS solution at pH 3. Each solution was injected into the chromatographic system ($n = 6$), and the average value of the peak areas was plotted against the concentrations. Curves were adjusted for linear regression with the least mean squares method. All the calibration plots in the concentration range studied were linear and with correlation coefficients (r^2) higher than 0.9997, as shown in Table 1.

Precision and Accuracy

In order to determine the intra-day precision and accuracy of the method, four known concentrations (0.5, 5, 25, and $50\,\mu g/mL$) of each drug were analysed on the same day ($n = 6$). Inter-day precision and accuracy were also evaluated over five consecutive days by performing six successive injections each day of the same concentrations. The results are

TABLE 1 Linear Regression Data and the Limits of Detection (LOD) and Quantification (LOQ) for PIP, LEV, NOR, and MOX

Analyte	Slope ± SD	Intercept ± SD	R^2	LOD (ng/mL)	LOQ (ng/mL)
Pipemidic Acid	2.67 ± 0.19	-0.27 ± 0.16	0.9998	8	28
Levofloxacin	0.83 ± 0.03	-0.149 ± 0.021	0.9997	56	171
Norfloxacin	2.47 ± 0.14	-0.09 ± 0.07	0.9999	33	100
Moxifloxacin	0.405 ± 0.024	-0.0199 ± 0.016	0.9999	14	43

summarized in Table 2. The low variability and high precision of the results obtained in different days are evident, which indicate the usefulness of the method.

Limits of Detection and Quantification

The limit of detection (LOD) and quantification (LOQ) for PIP, LEV, NOR, and MOX (n = 10) were determined with the 3.3s and 10s criterion, respectively, using a series of 10 solutions containing a low concentration. The results were based not only on the standard deviation of the response, but also on the slope of a specific calibration curve containing the analyte. Both LODs and LOQs are summarised in Table 1 according to the ICH harmonised tripartite guideline.[33] The values of the limits obtained were in the ng/mL range being sensitive enough for routine analysis.

Robustness

The robustness of the method was evaluated in terms of SDS (M), percentage of 1-propanol (%) (v/v), pH, percentage of thiethylamine (%) (v/v), and the flow rate of the mobile phase by six replicate injections of a standard solution at 5 μg/mL. The RSD (%) of the retention times

TABLE 2 Inter-Day and Intra-Day Precision and Accuracy of Analytes

Analyte	Concentration Added (μg/mL)	Found[a] (mean ± SD) (μg/mL)	Accuracy (%)	Intra-Day C.V. (%)	Found[b] (mean ± SD) (μg/mL)	Accuracy (%)	Inter-Day C.V. (%)
Pipemidic Acid	0.5	0.505 ± 0.008	1.6	2.1	0.501 ± 0.007	0.3	3.0
	5	4.97 ± 0.15	0.6	3.2	5.00 ± 0.08	0.1	5.1
	25	24.75 ± 0.13	1.0	0.5	24.7 ± 0.3	0.4	3.9
	50	50.17 ± 0.19	0.3	0.4	50.11 ± 0.18	0.2	5.2
Levofloxacin	0.5	0.4888 ± 0.0011	2.2	0.4	0.499 ± 0.010	0.2	2.8
	5	4.88 ± 0.08	2.3	1.7	5.03 ± 0.09	0.6	4.9
	25	24.51 ± 0.14	2.0	0.6	25.2 ± 0.4	0.7	6.4
	50	50.3 ± 0.6	0.6	1.0	50.16 ± 0.13	0.3	6.3
Norfloxacin	0.5	0.505 ± 0.006	1.0	0.1	0.498 ± 0.006	0.3	2.4
	5	4.90 ± 0.17	1.9	3.5	4.99 ± 0.08	0.1	4.7
	25	25.20 ± 0.06	0.8	0.3	24.9 ± 0.5	0.4	3.9
	50	49.9 ± 0.3	0.2	0.7	50.05 ± 0.13	0.1	5.4
Moxifloxacin	0.5	0.495 ± 0.007	1.0	1.4	0.496 ± 0.007	0.6	4.6
	5	4.89 ± 0.06	2.1	1.3	4.97 ± 0.03	0.6	4.3
	25	24.4 ± 0.3	2.3	1.2	24.85 ± 0.14	0.6	4.4
	50	50.1 ± 0.9	0.2	1.8	50.15 ± 0.14	0.3	4.3

[a]n = 6, [b]n = 5.

calculated from these variations is shown in Table 3 and was lower than 11.0%. Variation of the flow rate values (0.9, 1, 1.1 mL/min) had a stronger influence on the retention of the studied compounds than other parameters. However, the variations in all the parameters had no significant effect on resolution, peak area and peak shape.

Analysis of Pharmaceutical Formulations

The contents of nineteen pharmaceutical formulations, commercially available in Spain, were determined. Calibration curves were constructed by measuring the areas of the chromatographic peaks of the duplicate injections of the PIP, LEV, NOR, and MOX solutions at five increasing concentrations in the 1–25 μg/mL range. For each drug, six injections were

TABLE 3 Evaluation of the Robustness of the MLC Method

		t_R (min)			
Chromatographic changes	Level	PIP	LEV	NOR	MOX
A: Flow rate (mL/min)					
0.9	−0.1	7.7	7.4	11.3	10.23
1	0	7.6	6.7	10.08	9.06
1.1	+0.1	6.7	6.10	9.18	8.24
Mean ± SD		7.7 ± 0.7	6.7 ± 0.7	10.2 ± 1.0	9.2 ± 1.0
RSD (%)		9.7	10.0	10.3	10.9
B: SDS concentration (M)					
0.145	−0.05	7.7	6.8	10.3	9.23
0.15	0	7.6	6.7	10.08	9.06
0.155	+0.05	7.6	6.7	10.06	9.08
Mean ± SD		7.64 ± 0.03	6.76 ± 0.08	10.17 ± 0.16	9.13 ± 0.09
RSD (%)		0.34	1.12	1.6	1.01
C: Percentage of propanol (v/v)					
2.4	−0.1	8.3	7.3	11.20	9.9
2.5	0	7.6	6.7	10.08	9.06
2.6	+0.1	6.07	6.7	10.0	9.3
Mean ± SD		7.8 ± 0.4	6.9 ± 0.3	10.4 ± 0.7	9.3 ± 0.5
RSD (%)		5.21	4.8	6.4	5.7
D: pH of mobile phase					
2.9	−0.1	7.7	6.7	10.19	9.14
3	0	7.6	6.7	10.08	9.06
3.1	+0.1	7.7	6.7	10.21	9.11
Mean ± SD		7.65 ± 0.03	6.734 ± 0.021	10.16 ± 0.07	9.10 ± 0.04
RSD (%)		0.3	0.3	0.7	0.4
E: Percentage of TEA (v/v)					
0.45	−0.05	7.7	6.7	10.18	9.10
0.5	0	7.6	6.7	10.08	9.06
0.55	+0.05	7.6	6.7	10.11	9.02
Mean ± SD		7.63 ± 0.03	6.721 ± 0.014	10.13 ± 0.05	9.06 ± 0.04
RSD (%)		0.4	0.2	0.5	0.5

performed using 10 μg/mL of each compound. Figure 3 illustrates the chromatograms of the pharmaceuticals: pipemidic acid (a), levofloxacin (b), norfloxacin (c), and moxifloxacin (d). The excipients were eluted with the dead time or did not absorb at the measuring wavelength.

The labelled composition of the formulations, recoveries, and CV (%) values are shown in Table 4. The label claim percentage values were in the 96–106% range and the coefficient of variation in the range of 0.2–1.8%. As observed, the results obtained are in accordance with the labelled values.

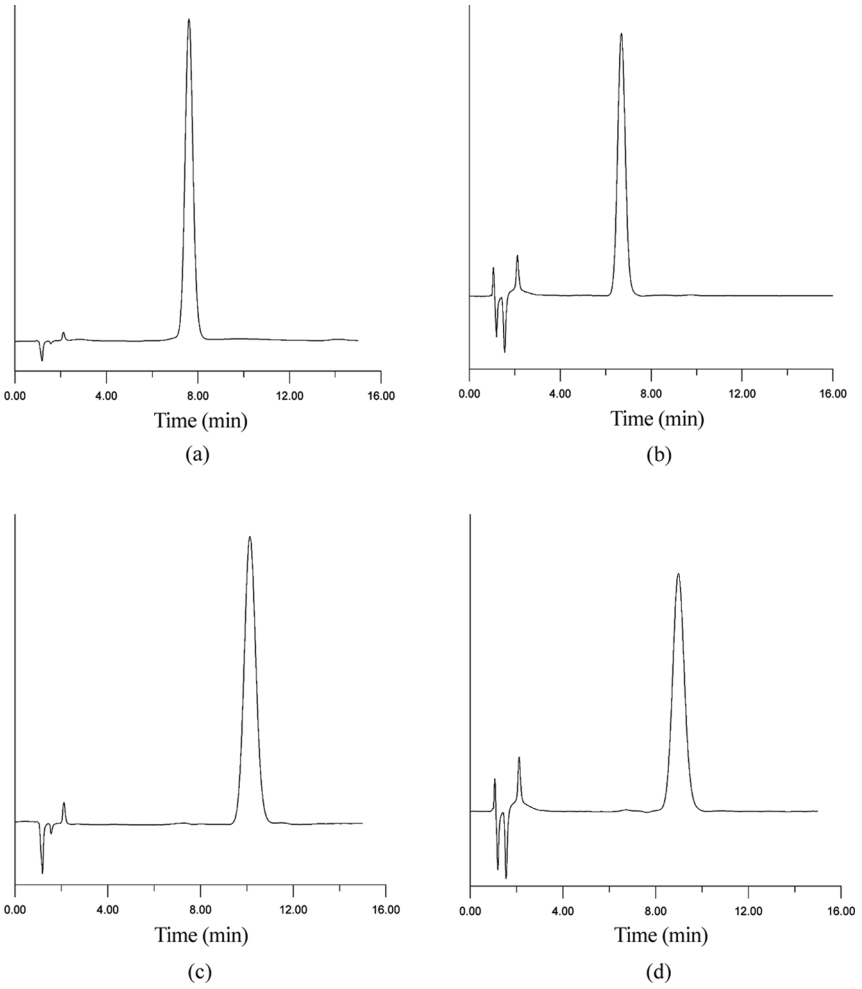

FIGURE 3 Chromatogram of (a) pipemidic acid (Nuril, 400 mg), (b) levofloxacin (Stada, 500 mg), (c) norfloxacin (Sadoz, 400 mg), and (d) moxifloxacin (Actira, 400 mg) pharmaceutical application (10 μg/mL). Mobile phase: 0.15 M SDS −2.5% 1-propanol −0.5% thiethylamine – pH 3, flow rate: 1 mL/min, UV detection at 276 nm.

TABLE 4 Recoveries of Pharmaceutical Formulations (n = 6)

Pharmaceutical (Laboratory)	Composition (mg)	Found (mg)	Label Claim (%)	C.V. (%)
Urisan	Per capsule: Pipemidic acid (400), excipients	415 ± 7	103.7	1.7
Galusan	Per capsule: Pipemidic acid (400), excipients	420 ± 8	104.9	1.8
Nuril	Per capsule: Pipemidic acid (400), excipients	421 ± 4	105.3	0.8
Normon	Per tablet: Levofloxacin (500), excipients	500.0 ± 1.7	100.0	0.3
Stada	Per tablet: Levofloxacin (500), excipients	485 ± 3	96.8	0.6
Tavanic	Per tablet: Levofloxacin (500), excipients	483.7 ± 1.0	96.7	0.21
Amicrobin	Per capsule: Norfloxacin (400), excipients	417.6 ± 1.3	104.4	0.3
Stada	Per tablet: Norfloxacin (400), excipients	407.0 ± 1.8	101.7	0.4
Nalion	Per tablet: Norfloxacin (400), excipients	423.7 ± 1.9	105.9	0.4
Uroctal	Per tablet: Norfloxacin (400), excipients	422.2 ± 1.3	105.5	0.3
Noroxin	Per tablet: Norfloxacin (400), excipients	383.9 ± 1.7	96.0	0.4
Norflok	Per tablet: Norfloxacin (400), excipients	420.3 ± 0.7	105.1	0.17
Normon	Per tablet: Norfloxacin (400), excipients	393.5 ± 0.6	98.4	0.6
Sadoz	Per tablet: Norfloxacin (400), excipients	393 ± 4	98.3	1.14
Bexal	Per tablet: Norfloxacin (400), excipients	421.0 ± 1.5	105.3	0.4
Esclebin	Per tablet: Norfloxacin (400), excipients	384.7 ± 1.8	96.2	0.5
Octegra	Per tablet: Moxifloxacin (400), excipients	394.4 ± 1.9	98.6	0.5
Proflox	Per tablet: Moxifloxacin (400), excipients	406.8 ± 1.6	101.7	0.4
Actira	Per tablet: Moxifloxacin (400), excipients	413 ± 6	103.3	1.3

CONCLUSIONS

The analytical method developed can be used to simultaneously separate and quantify an antibiotic mixture consisting of pipemidic acid, levofloxacin, norfloxacin, and moxifloxacin. Good sensitivity, linearity, and robustness were obtained. RSD values were lower than 2% and 6.4% for intra- and inter-day analyses, respectively. The recoveries in the

pharmaceutical samples were within a range of 96–106%, and no interferences from excipients were observed.

The proposed method is fast, precise, accurate, sensitive, and efficient, and the pharmaceutical formulation of the individual antibiotics studied in this research work can be routinely analysed.

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