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Short communication

The simultaneous separation and determination of five quinolone antibotics using isocratic reversed-phase HPLC: Application to stability studies on an ofloxacin tablet formulation

Leroy A. Shervington*, Michael Abba, Bushra Hussain, James Donnelly

University of Central Lancashire, Department of Biological Sciences, Preston PR1 2HE, UK

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Abstract

A rapid and reliable HPLC method was developed for the simultaneously separation and quantitation of five quinolones antibiotics; nalidixic acid, norfloxacin, ofloxacin, ciprofloxacin and lomefloxacin. All five tablet formulations of individual quinolone antibiotics were routinely assayed without interference. The calibration curves were linear ($r^2 \geq 0.999$) over the concentration range of 1.20–4.8 mg/100 ml. Selectivity, precision, sensitivity and accuracy were established and the method is stability indicating with respect to ofloxacin. The limit of detection and quantitation for ofloxacin was 18 and 36 μg/100 ml, respectively. The separation was performed on a Phenomenex ODS C18 column using an isocratic, ion-pairing mobile phase consisting of 35% (v/v) aqueous acetonitrile together with tetrabutylammonium acetate, sodium dodecyl sulphate and citric acid (pH* 3.4). All analyses were conducted at ambient temperature and was monitored using a Diode Array UV/VIS detector set at wavelengths 235, 254, 275 and 300 nm.

Keywords: Antibiotics; Nalidixic acid; Ofloxacin; Norfloxacin; Ciprofloxacin; Lomefloxacin; Isocratic reversed-phase system; Stability indicating

1. Introduction

Since the discovery of nalidixic acid [1], a number of structural modifications to the quinolone nucleus have been made in order to increase the antimicrobial activity and to enhance the pharmacokinetic performance of these drugs. Major findings relating to antibiotic development occurred during the 1980s with the realization that compounds with a fluorine atom at position C-6 and a piperazine or methylpiperazine at position C-7 such as norfloxacin, ciprofloxacin, ofloxacin and lomefloxacin, exhibit a broad spectrum of activity against Gram-positive and Gram-negative bacteria [2,3]. The general structure of fluoroquinolone antibacterial agents consists of a 1-substituted-1,4-dihydro-4-oxypyrine-3-carboxylic moiety combined with an aromatic or heteroaromatic ring (Fig. 1).

A number of analytical methods have been developed for analysing fluoroquinolones in both biological fluids and pharmaceutical formulations however; some of these methods have been found to be cumbersome, possessing poor precision and specificity and are uneconomical [4]. Due to the increased demand for reliability most researchers in the field now utilize HPLC because of its specificity, sensitivity, rapidity and robustness (Fig. 1).

There have been numerous reports describing the analysis of single and various combinations of fluoroquinolones in biological fluids, foods and environmental samples using either UV or fluorescence as the method of detection [5–16]. A HPLC method describing the simultaneous separation of six fluoroquinolones used fluorescence as the method of detection and was validated in order to determine the concentration of levofloxacin in biological fluids using UV as the method of detection [17]. A more recent report describes the simultaneous separation of nine fluoroquinolones from biological and environmental samples using fluorescence as the

* Corresponding author. Tel.: +44 1772 893519; fax: +44 1772 892929.
E-mail address: lashervington@uclan.ac.uk (L.A. Shervington).

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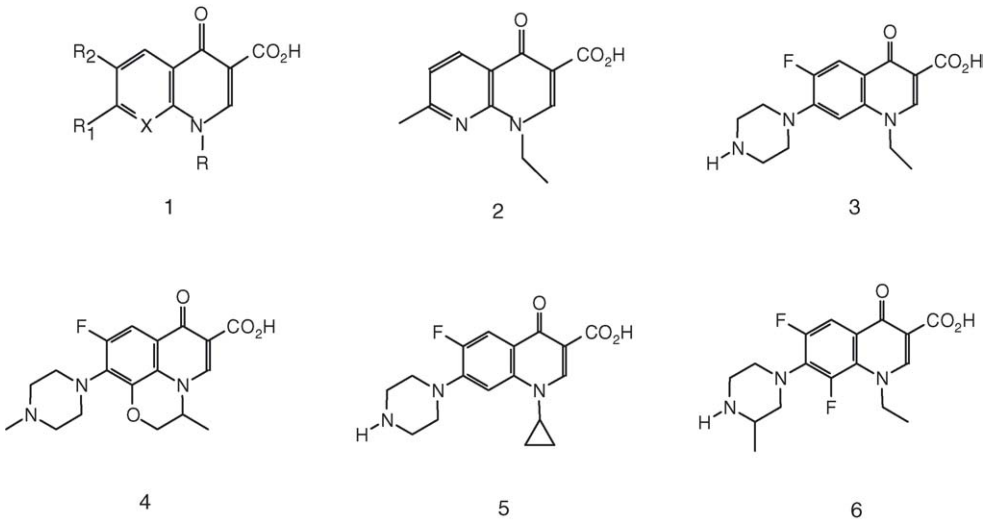

Fig. 1. The general structure of quinolone antibiotics (labelled 1). X, C; R, cyclopropyl, ethyl, fluoroethyl, methylamino, fluorophenyl group and thiazine or oxazine ring. R_1, piperazin-1-yl, 4-methylpiperazin-1-yl, 3-methylpiperazin-1-yl; R_2, fluorine. The quinolone antibiotics labelled 2–6 are nalidixic acid, norfloxacin, ofloxacin, ciprofloxacin and lomefloxacin, respectively.

method of detection, however, the separation was achieved using capillary electrophoresis [18].

Our investigation involved the modification of the method described above involving the separation of six fluoroquinolones in order to simultaneously separate and quantitate nalidixic acid, norfloxacin, ofloxacin, ciprofloxacin and lomefloxacin (Fig. 1) raw materials, and to carryout routine analysis of tablet formulations of these antibiotics. In addition, the method was further validated as a reliable stability indicating method for the analysis of ofloxacin tablets.

Although a combination of these actives would not normally be present in the same tablet formulation, it could provide a useful method for laboratories involved in the routine analysis of these antibiotics.

2. Materials and methods

2.1. HPLC instrumentation

The two HPLC systems used in the study consisted of: a Perkin-Elmer Series 410 Bio LC pump coupled to a Diode array UVD 340S Dionex detector and a Dell Optiplex GX1 computer with a Chomeleon Version 6.10 software package. The printer was a Hewlett Packard DeskJet 710C; a Varian Vista 5000 Liquid Chromatograph pump, coupled to a Waters 486 Tunable Absorbance UV detector and a Hewlett-Packard HP3395 integrator.

2.2. Chemicals

HPLC grade water and acetonitrile were purchased from Riedel-dettaen, dodecyl sulphate, sodium salt 98% (SDS), citric acid 99.5% and tetrabutylammonium acetate 97% (TBAA) were purchased from Aldrich. Nalidixic acid was purchased from Sigma. Ciprofloxacin, norfloxacin and lomefloxacin and excipients (microcrystalline cellulose, methylcellulose, magnesium stearate, hydroxypropylcellulose, lactose, maize starch, hypromellose croscarmellose, sodium starch glycollate, titanium dioxide, carnauba wax, propylene glycol, iron oxide, povidone and talc), were a generous gift from the Jordanian Pharmaceutical Manufacturing Company (Jordan). The ofloxacin was kindly provided by the United Pharmaceutical Manufacturing Company (Jordan). All the actives were of USP grade. Nalidixic acid tablets (500 mg) were manufactured by Sanofi Synthelabo (UK), norfloxacin tablets (400 mg) were manufactured by Merck Sharp and Dohme (UK), ofloxacin tablets (200 mg) were manufactured by Aventis Pharma Ltd. (UK), ciprofloxacin tablets (250 mg) were manufactured by Bioglan Generics (Ireland) and lomefloxacin tablets (400 mg) were manufactured by Pharmacia (Sweden). All the five tablets were purchased from The Royal Hospital Pharmacy, Preston (UK).

2.3. Chromatography

The chromatography was performed in the reversed-phase mode using a Phenomenex ODS C18(2), 150 mm × 4.6 mm i.d. column, linked to a Phenomenex ODS C18(2), 30 mm × 4.6 mm guard column. Both columns consisted of particle sizes equivalent to 5 μm. Manual injections were carried out using a Rheodyne model 9125 injector with a 20 μl loop. The mobile phase was 35% (v/v) aqueous acetonitrile consisting of 10 mM tetrabutylammonium acetate (TBAA), 10 mM sodium dodecyl sulphate and 25 mM citric acid. The pH* of the mobile phase was 3.4. Analysis was performed only after the column had reached equilibrium (approximately 1.5 h at a flow rate of 1 ml/min). The final flow

rate was set to 1.3 ml/min and the diode array detector was set to monitor at wavelengths 235, 254, 275 and 300 nm. All the analyses were performed at ambient temperature (25 °C).

2.4. Preparation of stock solutions

Stock solutions of antibiotics were prepared by individually weighing out 30 mg of each antibiotic, transferring them to 200 ml volumetric flasks, dissolving them in 35% (v/v) aqueous acetonitrile to give stock solutions of concentration equal to 15 mg/100 ml.

2.5. Calibration standards

Appropriate dilutions of the standard solutions (15 mg/100 ml) were prepared using 35% (v/v) aqueous acetonitrile to obtain concentrations equal to 1.2, 1.8, 3.0, 4.2 and 4.8 mg/100 ml. Regression analysis of the calibration data was then carried out.

2.6. Sample preparations

The stock solution (5 ml) of each of the antibiotics was transferred separately to 25 ml volumetric flasks and made upto the mark with 35% (v/v) aqueous acetonitrile to give a final concentration of 3 mg/100 ml.

2.7. Repeatability

Repeatability was carried out by preparing, separately one solution of each of the antibiotics solutions at a final concentration of 3 mg/100 ml together with a freshly prepared standard of the same concentration and injecting each solution six times. The percent recovery for the antibiotics was determined. The simultaneous analysis of all five antibiotics, each at a final concentration of 3 mg/100 ml was also checked for repeatability and were analysed accurately without interference.

2.8. Reproducibility

Reproducibility was carried out on separate days, which involved preparing six solutions of antibiotics at a final concentration of 3 mg/100 ml together with a freshly prepared standard and the percent recovery for the antibiotics were determined. The simultaneous analysis of the antibiotics, each at a final concentration of 3 mg/100 ml was also checked for reproducibility and were analysed accurately without interference.

2.9. Limits of quantitation and detection

The limit of quantitation and detection was determined for ofloxacin in order to accurately monitor its stability. It was achieved by preparing a solution of the antibiotic at 15 mg/100 ml using the standard procedure and then analysing the solutions after carrying out systematic dilutions.

2.10. Sample preparation (dosage form)

The assay of the five tablets was carried out in the following manner using standard extraction procedures. Each tablet formulation was treated in a similar manner apart from a minor modification required for lomefloxacin tablets, where the film coating had to be removed before powdering (crushing) was carried out. Ten tablets were weighed, crushed and the powdered tablet equivalent to 30 mg of the antibiotic was transferred to a 200 ml volumetric flask and treated with 35% (v/v) aqueous acetonitrile (100 ml). After sonification and cooling, the volumetric flask was made upto the mark with 35% (v/v) aqueous acetonitrile and a portion of the suspension (20 ml) was centrifuged at 6000 rpm for 25 min. A portion of the supernatant (10 ml) was filtered through 0.45 μm membrane filter and filtrate (5 ml) was transferred to a 25 ml volumetric flask and made upto the mark with 35% (v/v) aqueous acetonitrile. The final concentration of the sample solutions were 3 mg/100 ml and standards of the same concentration were also prepared. The volumetric flasks were covered with aluminium foil to avoid possible photodegradation on standing.

2.11. Accelerated stability studies of ofloxacin

The stability of ofloxacin antibiotic was determined by subjecting it to alkaline, acidic, oxidative and photolytic conditions in order to accelerate conditions conducive to degradation. Ofloxacin was treated separately with 0.1 M sodium hydroxide and 0.1 M hydrochloric acid. Ofloxacin was also treated with an excess of sodium periodate in a ratio of 1:5, respectively, and with a solution of hydrogen peroxide, the final concentration of hydrogen peroxide was 0.3% (w/v). The prepared solutions were transferred to amber glass screw top bottles and stored at 85 °C for 96 h. The solutions were then analysed using the established HPLC method.

Four conditions were used to assess the feasibility of the method to detect photolytic degradation. The first condition involved subjecting ofloxacin (in the powdered form) to natural daylight for a period of 240 h in sealed quartz cuvettes. The second condition involved subjecting the exposed powdered ofloxacin to direct UV light for 24 h. The third condition involved subjecting the exposed powdered tablet to direct UV light for 24 h. The final condition involved subjecting an aqueous solution of ofloxacin in sealed quartz cuvettes to UV light for 24 h. The sealed quartz cuvettes were positioned centrally in an enclosed UV light source generated by 12 TL 8W UV lamps linked to a multilamp power unit. The UV radiation used was continuous at a wavelength equal to 350 ± 10 nm. Although the theoretical assay concentration (3 mg/100 ml) was used throughout the developmental stage, the stability studies for ofloxacin were performed at a con-

centration of 15 mg/100 ml after confirming linearity upto 20 mg/100 ml.

3. Results and discussion

Although BP and USP describe validated isocratic HPLC methods [19–22] for the analysis of some of the fluoroquinolones of interest, norfloxacin, ofloxacin and ciprofloxacin in particular, these methods require the use of ovens to conduct the analysis at elevated temperatures, which are not always available. Furthermore, the oven needs to be maintained at constant temperatures with negligible fluctuation in order to achieve reliable, reproducible results. In addition, the majority of HPLC methods published involving, for example norfloxacin and lomefloxacin antibiotics, describe fluorescence as the main method of detection [23,24]. Because these detectors are less commonly available in the majority of laboratories, one of our objectives was to investigate the reliability of UV detectors for the analysis of the fluoroquinolones of interest.

We decided to use the published method [17] describing the separation of six fluoroquinolones in order to analyse the five antibiotics of interest. After attempts to achieve separation of the five antibiotics using this method, it was found that overlap between norfloxacin and lomefloxacin prevented complete separation. In order to resolve the overlap, the concentrations of the SDS, TBAA and citric acid were kept constant while gradually decreasing the composition of acetonitrile from 43 to 35% in increments of 2%. However, the peaks corresponding to norfloxacin and lomefloxacin were not completely resolved at any solvent composition. Only after changing from a Waters Symmetry column to a Phenomenex column was complete separation achieved with elution times of 4.54, 7.85, 10.49, 11.10 and 11.92 min for nalidixic acid, ofloxacin, lomefloxacin, norfloxacin and ciprofloxacin, respectively (Fig. 2).

3.1. System suitability and linearity

System suitability was performed before conducting the determination for linearity. Mixtures of the five standard antibiotics at concentrations used to estimate linearity (1.2–4.8 mg/100 ml) were prepared and system suitability was confirmed after obtaining consistent determinations from six injections. The antibiotics were determined at concentrations ranging from 40 to 160% of the theoretical assay concentration and gave linear responses (Tables 1 and 2).

3.2. Repeatability and reproducibility

Repeatability and reproducibility of the test procedure using the theoretical assay concentration of 3 mg/100 ml for the five antibiotics was shown (Tables 3 and 4).

Table 1
The analysis nalidixic acid, lomefloxacin, norfloxacin, ofloxacin and ciprofloxacin by HPLC using concentrations ranging from 1.20 to 4.80 mg/100 ml

Nalidixic acid		Lomefloxacin		Norfloxacin		Ofloxacin		Ciprofloxacin	
Amount added (mg/100 ml)	% Recovery*	Amount added (mg/100 ml)	% Recovery*	Amount added (mg/100 ml)	% Recovery*	Amount added (mg/100 ml)	% Recovery*	Amount added (mg/100 ml)	% Recovery*
1.21	98.93	1.20	101.06	1.20	100.23	1.21	100.01	1.20	99.99
1.82	99.45	1.80	100.41	1.80	100.33	1.80	100.60	1.80	99.69
3.03	99.45	3.00	100.43	3.00	100.30	3.00	100.04	3.00	99.95
4.24	99.75	4.20	100.40	4.20	99.78	4.21	100.04	4.20	100.29
4.85	99.37	4.80	100.40	4.80	99.77	4.81	99.10	4.80	100.01
Mean	99.09		100.54		100.08		99.96		99.99
S.D.	0.709		0.290		0.282		0.539		0.213
R.S.D.	0.716		0.289		0.282		0.539		0.213

* Average of the two injections. Two sets of experiments were carried out using five concentrations; each set was assayed on separated days using freshly prepared solutions.

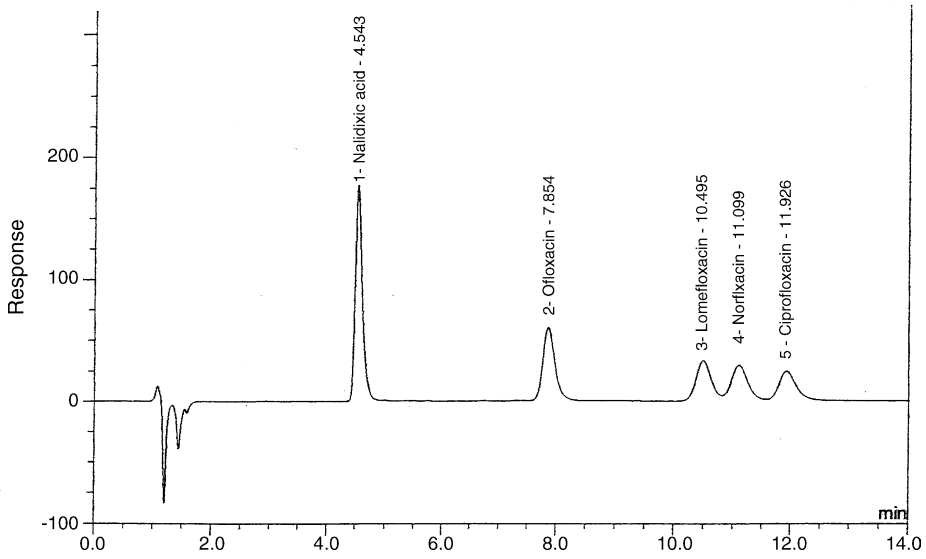

Fig. 2. The separation of the five quinolone antibiotics. The elution times of 4.54, 7.85, 10.49, 11.10 and 11.92 min correspond to nalidixic acid, ofloxacin, lomefloxacin, norfloxacin and ciprofloxacin, respectively.

Table 2
Regression analysis of the calibration data

Antibiotics	Slope	Intercept	Correlation coefficient
Nalidixic acid	0.010	−0.101	0.9994
Lomefloxacin	0.0206	−0.146	0.9998
Norfloxacin	0.0254	−0.176	0.9998
Ofloxacin	4.796	−0.660	0.9992
Ciprofloxacin	0.3737	0.031	0.9997

3.3. Limits of detection and quantitation for ofloxacin

The limit of detection and quantitation for ofloxacin was 18 and 36 μg/100 ml, respectively, and the R.S.D. for the LOQ ($n = 3$) was 3.21%.

3.4. Assay of tablets

The results of the assays obtained for tablets of nalidixic acid, norfloxacin, ofloxacin, ciprofloxacin and lomefloxacin were 100.49, 100.41, 98.29, 100.98 and 99.63%, respectively, with corresponding R.S.D.'s of 0.46, 0.35, 0.48, 0.23 and 0.62, respectively.

3.5. Stability studies

Ofloxacin proved to be relatively stable under both acidic and alkaline conditions resulting in only minor degradation, however when ofloxacin was subjected to oxidative conditions using either sodium periodate or hydrogen peroxide, complete degradation was observed. When ofloxacin was exposed to natural daylight (240 h) and direct UV light (24 h), the recoveries were 80 and 85%, respectively. In comparison, when the powdered tablet was exposed to direct UV light (24 h), there was a decrease in stability of the ofloxacin affording an assay of 75%. This would infer that one or more of the excipients might have contributed to the increase in degradation. When an aqueous solution of ofloxacin was subjected to direct UV light (24 h), there was only a 36% recovery. For-

Table 3
Repeatability of the test procedure

	Nalidixic acid 3.005 mg/100 ml	Lomefloxacin 3.010 mg/100 ml	Norfloxacin 3.024 mg/100 ml	Ofloxacin 3.005 mg/100 ml	Ciprofloxacin 3.008/100 ml
	99.46	101.63	99.84	98.61	99.19
	98.99	100.88	100.35	100.43	101.82
	101.30	100.49	100.65	99.60	100.40
	100.14	100.20	99.01	100.10	100.74
	99.80	98.40	99.78	100.98	99.90
	100.87	99.61	99.84	100.23	100.14
Mean	100.09	100.20	99.74	99.99	100.36
S.D.	0.87	1.11	0.714	0.812	0.883
R.S.D.	0.87	1.10	0.717	0.812	0.880

The determinations are shown as % recoveries.

Table 4
Reproducibility of the test procedure

Nalidixic acid		Lomefloxacin		Norfloxacin		Ofloxacin		Ciprofloxacin	
Amount added (mg/100 ml)	% Recovery*	Amount added (mg/100 ml)	% Recovery*	Amount added (mg/100 ml)	% Recovery*	Amount added (mg/100 ml)	% Recovery*	Amount added (mg/100 ml)	% Recovery*
3.010	100.15	3.011	97.74	3.015	99.94	3.008	98.91	3.010	100.13
3.000	99.66	3.012	98.50	3.006	100.70	3.007	98.95	3.003	98.03
3.015	99.60	3.005	100.57	3.008	99.24	3.013	96.91	3.003	97.00
3.007	99.80	3.009	102.35	3.023	98.69	3.005	100.32	3.000	101.63
3.002	98.53	3.013	101.05	3.020	101.24	3.012	98.65	3.014	98.48
3.005	99.02	3.023	97.31	3.033	99.45	3.010	102.06	3.011	101.62
Mean	99.46		99.59		99.88		99.30		99.48
S.D.	0.584		2.025		0.952		1.730		1.942
R.S.D.	0.588		2.034		0.953		1.740		1.950

*Average of the two injections.

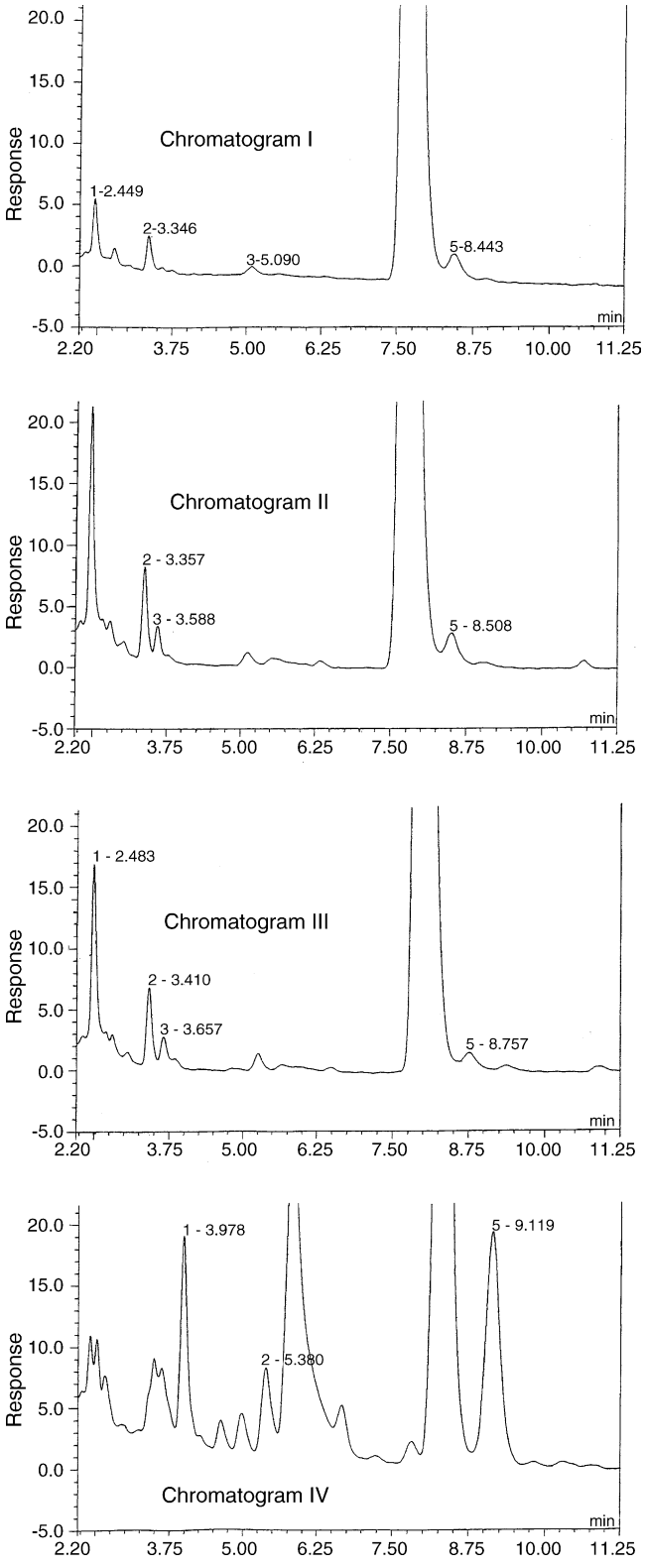

Fig. 3. Chromatogram I shows the partial degradation of powdered ofloxacin exposed to natural light for 240 h. Chromatogram II shows partial degradation of powdered ofloxacin exposed to direct UV light for 24 h. Chromatogram III shows the partial degradation of powdered ofloxacin tablets exposed to direct UV light for 24 h. Chromatogram IV shows the partial degradation of an aqueous solution of ofloxacin exposed to direct UV light for 24 h.

Table 5
The analysis of ofloxacin under various storage conditions

Conditions	% Recovery of ofloxacin
1	97
2	93
3	Complete degradation
4	Complete degradation
5	80
6	85
7	75 (% assay)
8	36

The stressed conditions used for testing stability. (1) Ofloxacin dissolved in 0.1 M hydrochloric acid and stored at 85 °C for 96 h. (2) Ofloxacin dissolved in 0.1 M sodium hydroxide and stored at 85 °C for 96 h. (3) Ofloxacin dissolved in aqueous solution of sodium periodate and stored at 85 °C for 96 h. (4) Ofloxacin dissolved in 0.3% hydrogen peroxide and stored at 85 °C for 96 h. (5) Powdered ofloxacin exposed to natural daylight for 240 h. (6) Powdered ofloxacin exposed to direct UV light for 24 h. (7) Powdered ofloxacin tablets exposed to direct UV light for 24 h. (8) Ofloxacin dissolved in water and exposed to direct UV light for 24 h.

tunately, the detected degradation products produced from the accelerated stability studies were resolved from the peak corresponding to the ofloxacin (Fig. 3 and Table 5).

4. Conclusions

The analytical method can be used to simultaneously separate and quantitate an antibiotic mixture consisting of nalidixic acid, ofloxacin, norfloxacin, ciprofloxacin and lomefloxacin. Tablet formulations of the individual antibiotics studied in this investigation can be routinely analysed with accuracy and precision, and the method can be described as stability indicating with respect to ofloxacin. Further developmental work would need to be carried out to see whether the method could be used to analyse the fluoroquinolones in biological fluids.

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