Available online at www.sciencedirect.com
SCIENCE DIRECT
Talanta 68 (2006) 1215–1221
Talanta
www.elsevier.com/locate/talanta

Determination of fluoroquinolones in urine and serum by using high performance liquid chromatography and multiemission scan fluorimetric detection

A. Espinosa-Mansilla*, A. Muñoz de la Peña, D. González Gómez, F. Salinas López

Department of Analytical Chemistry, University of Extremadura, Avd. Elvas s/n, Badajoz 06071, Spain

Received 26 January 2005; received in revised form 9 June 2005; accepted 15 July 2005
Available online 25 August 2005

Abstract

A high performance liquid chromatography (HPLC) method has been developed for the simultaneous determination of four fluoroquinolones. The studied compounds have been enoxacin (ENO), norfloxacin (NOR), ofloxacin (OFLO) and enrofloxacin (ENRO). An isocratic elution method, using a mixture of tetrahydrofuran (8%) and phosphate buffer (pH 3.00, 30.0 mM, 92%) as mobile phase, has been developed. Fluorimetric detection, exciting at 277 nm, and multiemission scan (407 nm for ENO, 444 nm for both NOR and ENRO and 490 nm for OFLO) has been used. Detection limits of 500, 14.7, 25.2 and 15.0 ng mL^{-1} for ENO, NOR, OFLO and ENRO, respectively, have been obtained. The proposed method has been satisfactorily applied to analyze NOR, OFLO and ENRO in human urine and serum samples.

Keywords: Fluoroquinolones; Enoxacin; Norfloxacin; Ofloxacin; Enrofloxacin; HPLC; Serum; Urine

1. Introduction

Quinolones are an important group of synthetic antibiotics with antibacterial action, but the introduction of the fluorinated quinolones represents important therapeutic advantages, because this antibiotic group shows higher antibacterial activity and convenient pharmacokinetic profiles [1]. These compounds have a carboxylic acid group in position 4, and are frequently referred to as 4-quinolones. Their antibacterial activity increased by the addition of 6-fluoro- and 7-piperazinyl groups to the molecule. The main uses, is in the treatment of human and veterinary diseases, and are very useful in preventing diseases in animals [2–5]. The therapeutic levels are on the order of 5 mg L^{-1}. Low amounts of fluoroquinolones are found in plasma and their main excretion pathway in urinary. Because of the possible development of resistance of human pathogens to antibiotics, there is nowadays concern about the possibility of exposure to low levels of these compounds [6].

High performance liquid chromatography (HPLC) has become an important tool for routine determination of fluoroquinoloness [7]. Several references about the determination of different fluoroquinolones in biological fluids, by using HPLC with UV and/or fluorescence detection, have been recently reported [8–10]. Novel fluoroquinolones, as moxifloxacin, have been also analyzed using HLC in combination with column switching techniques [11]. Chromatography coupled to pneumatically assisted electrospray ionization mass spectrometry has been reported to analyze NOR, CIPRO and OFLO in human urine, the detection limit being 10 ng mL^{-1} [12]. Recently, Ferdig et al. [13] developed a capillary electrophoresis (CZE) method for nine fluoroquinolones, with laser fluorescence detection, in biological and environmental samples.

In the past decade, multivariate techniques have been incorporated to the analytical protocols [14]. Several chemometric methodologies have been employed for the simultaneous determination of fluoroquinolones. In

* Corresponding author. Tel.: +34 924289375; fax: +34 924289375.
E-mail address: nuncy@unex.es (A. Espinosa-Mansilla).

0039-9140/$ – see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.talanta.2005.07.035

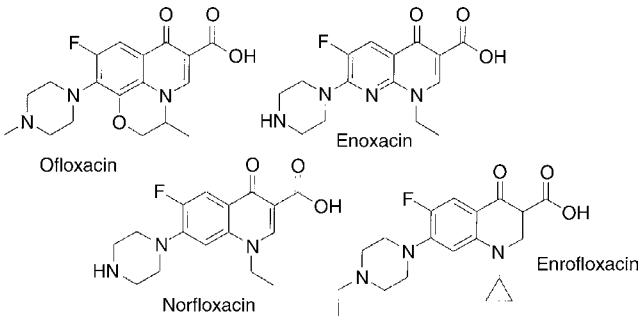

Fig. 1. Chemical structures of the fluoroquinolones herein studied.

particular, full spectrum multivariate calibration methods, offering the advantage of their speed, because the separation steps may be avoided. Recently, ENO, NOR and OFLO have been simultaneously determined in human serum, by using three way fluorescence data and parallel factor analysis (PARAFAC), and exploiting the so called "second order advantage" to calibrate analytes in the presence of any number of uncalibrated (serum) components [15]. Also, these fluoroquinolones can be simultaneously determined in urine by using the native fluorescence emission in micellar medium and partial least squares (PLS) calibration [16]. However, the simultaneous determination of ENRO and NOR, by using multivariate calibration, is not possible due to identical luminescence spectral characteristics of these two fluoroquinolones.

In this paper, an HPLC method for the determination of enoxacin (ENO), norfloxacin (NOR), ofloxacin (OFLO) and enrofloxacin (ENRO) has been developed (Fig. 1). The determination of NOR, ENRO and OFLO in serum and urine is proposed in a single-run analysis. The compounds are quantified using multiemission scan fluorimetric detector.

2. Experimental

2.1. Apparatus

The chromatographic studies were performed on a Hewlett-Packard Mod. 1100 LC instrument, equipped with degasser, quaternary pump, manual six-way injection valve, containing a 20 μL loop, multi scan fluorimetric detector (Agilent 1100 fast-scanning detector) and CHEMSTATION software package to control the instrument, data acquisition and data analysis. Acquisition of excitation and emission spectra: scan rate 28 ms per point. The fluorimetric detector is equipped with a 8 μL flow cell. An analytical column Nova-Pak C_{18}, 150 mm × 3.9 mm length, 4 μm particle size and 60 A pore size (Waters Millipore) was used. Emission wavelength at 407, 444 and 490 nm were selected for ENO, both NOR and ENRO, and OFLO, respectively, using excitation at 277 nm.

2.2. Mobile phase

The mobile phase was formed by a mixture of 92% phosphate buffer and 8% tetrahydrofuran (THF), using a flow rate of 1 mL min^{-1}.

To prepare the pH 3.0 buffer solution, di-potassium hydrogen phosphate trihydrate (Merck) was dissolved in ultrapure water, which was obtained from a Millipore Milli-Q system. The acidity was fixed using HCl (Merck) at pH 3.0. The buffer concentration was 30.0 mmol L^{-1}.

2.3. Reagents and solutions

All solvents used were gradient grade for liquid chromatography (Merck). Enoxacin, ofloxacin, norfloxacin and enrofloxacin were purchased from Sigma–Aldrich (Spain), purity >98%. Standard solutions of each compound (100 μg mL^{-1}) were prepared by dilution in ethanol (avoiding exposure to direct light and maintained at 4 °C being stable at least during 1 week).

3. Results and discussion

3.1. Selection of the chromatographic parameters

The emission spectra of the studied compounds are reported in Fig. 2. The detection was performed with a fast-scanning fluorimetric detector. The excitation was fixed at 277 nm. This wavelength was optimized for the simultaneous detection. The emission maxima were located at 409 nm for ENO, 444 nm for NOR and ENRO, and 490 nm for OFLO. The possibility of using a multiemission

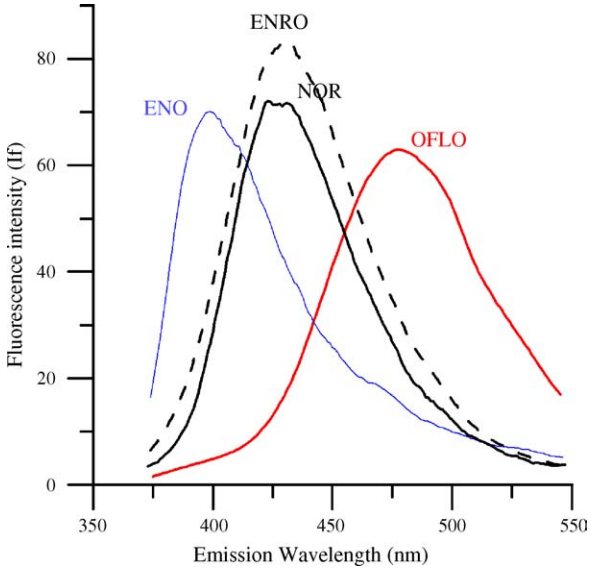

Fig. 2. Emission spectra of ENO, OFLO, ENRO and NOR, exciting at 277 nm.

Table 1
Chromatographic parameters obtained in the resolution of the fluoroquinolones, for different percentages of THF in the mobile phase

THF (%)	ENO		OFLO			ENRO			NOR		
	t_R	k'	t_R	k'	R_s	t_R	k'	R_s	t_R	k'	R_s
12	2.29	1.01	2.77	1.43	1.74	3.29	1.88	1.73	3.58	2.14	0.85
8	3.46	2.03	4.32	2.78	2.46	5.34	3.68	2.65	6.07	4.32	1.29
6	4.85	3.25	6.00	4.26	2.37	7.94	5.96	3.29	8.76	6.68	1.18

t_R, Retention time; k', capacity factor; R_s, resolution.

scan, allow us to use the appropriate emission wavelength and increase the sensitivity for each analyte.

For the selection of the mobile phase, different organic solvents were tested. Acetonitrile, methanol and THF were used to select the best one. When acetonitrile and methanol were assayed poor defined and non-symmetric peaks were recorded. However, THF provides very well defined and symmetric peaks and an adequate resolution in a short time. In acid medium (pH 2.5–3.0) the four analytes are eluted in about 5 min and adequate resolution is observed. When the pH of the mobile phase was increased pH >3.5, especially between ENRO and NOR the retention time decreased. The best peaks resolution was observed at the pH near to 3.0. In Table 1, the retention times, capacity factors and resolution values, for three different compositions of THF and buffer (pH 3), in the mobile phase, are summarized. The resolution increases when the THF content decreases. However, for ENRO and NOR, the resolution decreases for amounts shorter than 8% THF. On the other hand, the retention time is drastically affected by the THF content. A mobile phase, composed by 92% buffer phosphate (pH 3) and 8% THF, was selected as an adequate compromise.

In the optimized conditions for the mobile phase, good resolution of all peaks was obtained, and all the compounds were eluted in less than 7 min. The ENRO/NOR gave the poorest resolution of all the peaks. The possibility of using multiemission scan, provided by the fast-scanning fluorimetric detector, permits us to monitor each component at its most sensitive emission wavelength. In Fig. 3A, chromatograms obtained at several emission wavelengths are shown and, in Fig. 3B, the bi-dimensional projection plots of multiwavelength emission versus retention time, for the above chromatogram, when an excitation wavelength of 277 nm was used, is represented. The best sensitivity is attained by monitoring at 407, 490 and 444 nm, for ENO, OFLO and both ENRO and NOR, respectively.

3.2. Analytical parameters

Under the selected conditions, calibration graphs were obtained by preparing samples of the mixture of the four compounds in triplicate. The standard samples containing different volumes of stock fluoroquinolone solutions were prepared, in 25 mL volumetric flasks, and diluted with pH 3.0 buffer solutions to the mark. Previously to the injection, the samples were filtered thorough a 0.22 μm nylon filter. The study was performed in the concentration ranges up to 5000 ng mL^{-1}, up to 250 ng mL^{-1} and up to 200 ng mL^{-1} for ENO, OFLO and both ENRO and NOR, respectively, using the peak area as analytical signal. The regression

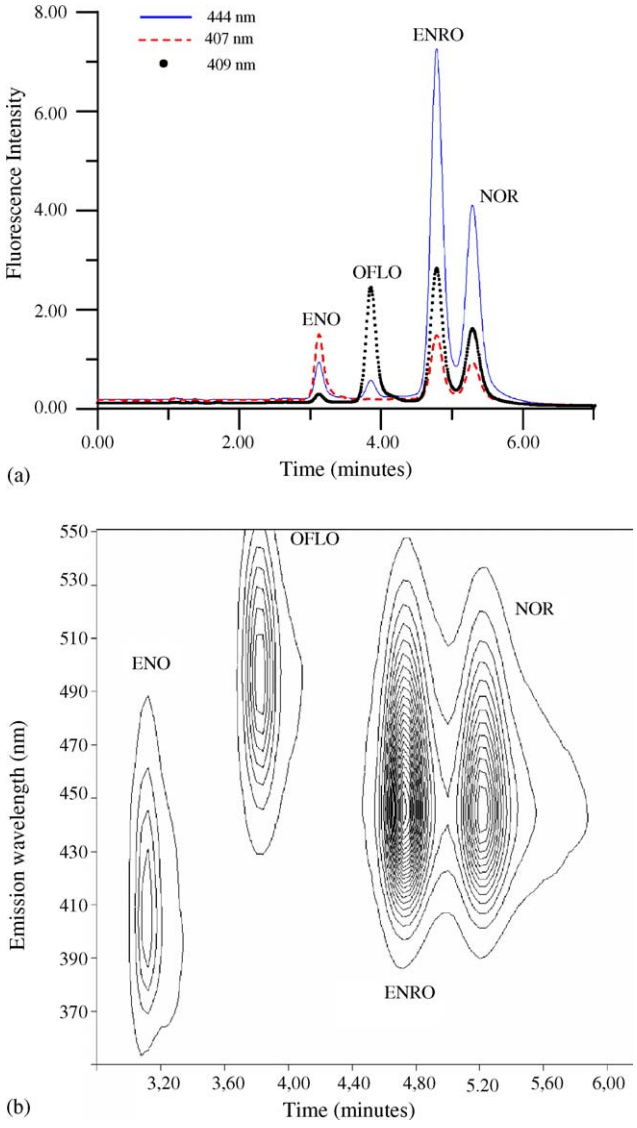

Fig. 3. (a) Chromatogram of a standard sample containing the four analytes: ENO (1 μg mL^{-1}), OFLO (0.25 μg mL^{-1}), ENRO (0.25 μg mL^{-1}) and NOR (0.1 μg mL^{-1}), exciting at 277 nm, and performing the multiemission scan at 407, 444 and 490 nm. (b) Bi-dimensional emission wavelength–time contour plot for the chromatogram above indicated.

Table 2
Statistical parameters for not weighted least squares regression of ENO, OFLO, ENRO and NOR, in the selected chromatographic conditions

Analytical signal: peak area	ENO	OFLO	ENRO	NOR
Intercept ($\pm\sigma$)	0.04 (0.006)	2.86 (1.07)	1.86 (1.58)	1.04 (0.36)
Slope ($\pm\sigma$)	0.021 (0.002)	0.18 (0.01)	0.49 (0.01)	0.23 (0.01)
(FU.s.mL ng^{-1})				
Regression coefficient (R)	0.996	0.995	0.998	0.998
S y/x	2.93	1.13	2.14	1.00
Linearity (%) [19]	97	97	98	98
Limit of detection (ng mL^{-1}) ($K = 3$) [16]	300	17.9	9.8	9.8
Limit of detection (ng mL^{-1}) ($\alpha = \beta = 0.05$) [17]	500	25.2	15.0	14.7
Sensitivity (1/γ) (ng mL^{-1}) [19]	100	6.3	4.4	4.3

FU, fluorescence units.

Table 3
Intraday results using successive injections of 10 individual samples containing 3000, 160 and 120 ng mL^{-1} of ENO, OFLO and both ENRO and NOR

	ENO	OFLO	ENRO	NOR
Calculated mean concentration $\pm\sigma$ (ng mL^{-1})	2890 (30)	152 (1.21)	119 (1.05)	119 (1.89)
R.S.D. (%)	1.0	0.8	0.9	1.6
t_R (min) $\pm\sigma$	3.44 ± 0.01	4.29 ± 0.02	5.40 ± 0.02	6.03 ± 0.03
$W_{1/2}$ (min) $\pm\sigma$	0.168 ± 0.001	0.187 ± 0.002	0.234 ± 0.002	0.257 ± 0.002
$R_s \pm\sigma$	–	2.40 ± 0.01	2.63 ± 0.01	1.27 ± 0.01

σ, standard deviation, $n = 10$.

statistical parameters are shown in Table 2. In addition, the detection limit value (LOD), according with Long and Winefordner [17] and Clayton et al. [18] criteria, linearity and analytical sensitivity [19] are also included. The intraday precision, expressed as percentage of relative standard deviation (R.S.D.), was studied by performing successive injections of 10 individual samples (Table 3). Good R.S.D. values are obtained for all the assayed fluoroquinolones.

3.3. Analysis of urine samples

The proposed method was applied to the determination of the cited fluoroquinolones in urine samples. In a 25 mL volumetric flask, 1 mL sample of fresh urine belonging to healthy people, were appropriated spiked with an appropriated volume of the stock ENO, OFLO, ENRO and NOR solution, simulating biological concentrations (50 μg mL^{-1}). The samples were maintained in the dark during 30 min, to favour the interactions between the analytes and the urine matrix, and to avoid the natural light effect, and then the samples were diluted with ultrapure water to the mark. In a 10 mL volumetric flask, 0.50 mL of the above fortified urine solution was placed, and diluted with pH 3.0 buffer solution to the mark. The urine suffers a dilution of 500-fold. In these conditions, ENO would not be analyzed in urine, simultaneously with NOR, OFLO and ENRO in a single run, because of the low sensitivity observed for this compound, in comparison with the other selected fluoroquinolones. Also, unspiked urine samples were prepared. All samples were injected by triplicate. In Fig. 4, chromatograms of spiked and unspiked urines are shown. To calculate the concentration of the three fluoroquinolones in the urine samples, and to check the effect of the presence of the urine matrix, the standard addition and the external standard method were employed. The comparison between the slopes obtained in both methods was statistically indistinguishable ($F_{cal} < F_{tab}$). In Table 4, these results are summarized.

In order to get further insight into the accuracy and precision of the method herein developed, linear regression analysis of the nominal versus found concentration values was applied. The estimated intercept and slope were compared with their ideal values 0 and 1, using the elliptical joint

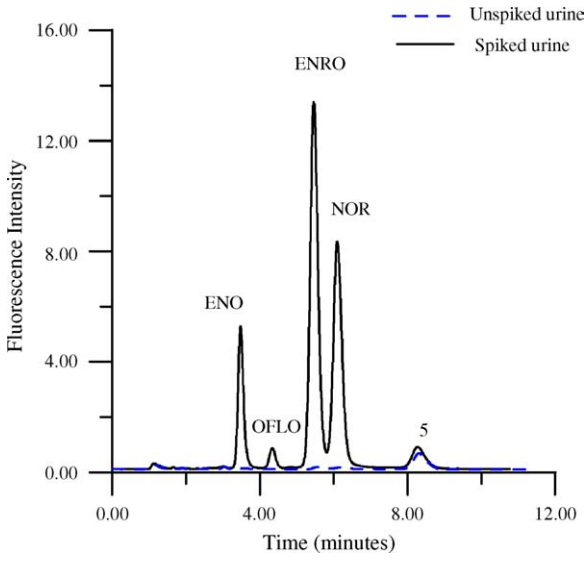

Fig. 4. Chromatogram of (----) unspiked urine sample, and (—) spiked urine with ENO (3 μg mL^{-1}), OFLO (0.16 μg mL^{-1}), ENRO (0.16 μg mL^{-1}) and NOR (0.16 μg mL^{-1}) (peak 5 corresponds to an unknown compound in the urine matrix), $\lambda_{excitation} = 277$ nm, $\lambda_{emission} = 444$ nm.

Table 4
Results obtained in the determination of OFLO, ENRO and NOR in human urine, fortified with 50 μg mL^{-1} of each fluoroquinolone, by using the standard addition and external standard methods

	Standard addition method (SAM)			
	Added[a] (ng mL^{-1})	Found[a] (ng mL^{-1})	REP (%)	Found vs. added regression
OFLO	80	77	−4	$[OFLO]_{found} = 0.93\ [OFLO]_{added}$, $R = 0.994$
	120	132	+10	
	160	166	+3	
	200	190	−5	
	Found[b] (SAM): 49 μg mL^{-1}			Found[b] (ESM): 45 μg mL^{-1}
	SAM–ESM slope comparison test: $t_{cal} = 0.471$, $t_{tab} = 2.32$			
NOR	40	35	−12	$[NOR]_{found} = 1.02\ [NOR]_{added}$, $R = 0.998$
	80	86	+7	
	120	131	+9	
	160	156	−3	
	Found[b] (SAM): 52 μg mL^{-1}			Found[b] (ESM): 51 μg mL^{-1}
	SAM–ESM slope comparison test: $t_{cal} = 0.380$, $t_{tab} = 2.60$			
ENRO	40	37	−8	$[ENRO]_{found} = 0.97\ [ENRO]_{added}$, $R^2 = 0.987$
	80	87	+9	
	120	126	+5	
	160	153	−4	
	Found[b](SAM): 46 μg mL^{-1}			Found[b] (ESM): 45 μg mL^{-1}
	SAM–ESM slope comparison test: $t_{cal} = 0.302$, $t_{tab} = 2.33$			

REP, relative error of prediction (%); ESM, external standard method; SAM, standard addition method.
[a] Referred to injected urine samples.
[b] Referred to original urine samples.

confidence region (EJCR) test [20]. Any point, which lies inside the EJCR, is compatible with the data at the chosen confidence level. If the point (0, 1) lies inside the EJCR, then bias is absent and, consequently, the recovery may be taken as unity (or 100% in percentile scale) (Fig. 5). The results indicate the lack of interference from the urine matrix. The repeatability study was performed using the injection of 10 individual fortified urine samples. The injected quantity for each fluoroquinolone was 3.2, 2.4 and 2.4 ng for OFLO, ENRO and NOR, respectively. The precision was expressed as relative standard deviation and adequate values were obtained in all cases (Table 5).

3.4. Analysis of serum samples

The proposed method was also applied to the determination of the cited fluoroquinolones in serum samples. Serum samples belonging to healthy people were appropriated spiked with ENO, OFLO, ENRO and NOR, simulating biological concentrations (1 μg mL^{-1}), and then maintained in the dark during 30 min, as indicated in the urine analysis. The dilution factor was 10 (1.0 mL of serum in 10 mL using buffer solution pH 3.0). Before the injection, the samples were centrifuged and filtered through a 0.22 μm nylon filter. With this dilution, the final concentration of each of the drugs is at the biological level. In these conditions ENO could not be analyzed in a single run because of its low sensitivity in comparison with the other fluoroquinolones. In Fig. 6, chromatograms of spiked and unspiked serum are shown. In all serum samples NOR, ENRO and OFLO were analyzed and recovery values between 96 and 112% were obtained. The standard addition method and the external standard method were used to calculate the concentration of the three analytes. The comparison between the slopes obtained in both methods is statistically

Table 5
Within day precision for OFLO, ENRO and NOR, in human urine and serum samples

	Urine			Serum		
	OFLO	ENRO	NOR	OFLO	ENRO	NOR
Injected quantity (ng)	3.2	2.4	2.4	2.0	2.0	2.0
R.S.D.* (%)	0.9	2.4	3.4	4.1	3.3	6.2
$W_{1/2}$ (min) ± σ	0.15 ± 0.001	0.170 ± 0.002	0.171 ± 0.003	0.177 ± 0.002	0.204 ± 0.003	0.212 ± 0.002
$R_s \pm \sigma$	2.8 ± 0.13	2.55 ± 0.15	1.03 ± 0.09	2.07 ± 0.08	2.41 ± 0.16	1.01 ± 0.98

* $n = 10$.

Table 6
Results obtained in the determination of OFLO, ENRO and NOR, in human serum fortified with 1 μg mL^{-1} of each fluoroquinolone, by using the standard addition and external standard methods

	Standard addition method (SAM)			
	Added[a] (ng mL^{-1})	Found[a] (ng mL^{-1})	REP (%)	Found vs. added regression
OFLO	80	86	+7	$[OFLO]_{found} = 0.99\ OFLO]_{added}$, $R = 0.998$
	120	122	+2	
	160	165	+5	
	Found[b] (SAM): 0.95 μg mL^{-1}			Found[b] (ESM): 0.81 μg mL^{-1}
	SAM–ESM slope comparison test: $t_{cal} = 0.091$, $t_{tab} = 2.54$			
NOR	40	45	+12	$[NOR]_{found} = 0.96\ [NOR]_{added}$, $R = 0.999$
	80	81	+1	
	120	121	0	
	160	159	−1	
	Found[b] (SAM): 0.98 μg mL^{-1}			Found[b] (ESM): 1.00 μg mL^{-1}
	SAM-ESM slope comparison test: $t_{cal} = 0.375$, $t_{tab} = 2.20$			
ENRO	40	45	+12	$[ENRO]_{found} = 0.92\ [ENRO]_{added}$, $R = 0.992$
	80	88	+10	
	120	115	−4	
	160	159	−1	
	Found[b] (SAM): 0.84 μg mL^{-1}			Found[b] (ESM): 0.94 μg mL^{-1}
	SAM–ESM slope comparison test: $t_{cal} = 0.017$, $t_{tab} = 2.44$			

REP, relative error of prediction (%); ESM, external standard method; SAM, standard addition method.
[a] Referred to injected urine samples.
[b] Referred to original urine samples.

indistinguishable ($F_{cal} < F_{tab}$). The results indicate the lack of interference of the serum matrix. In Table 6, these results are summarized. Similarly to the analysis of urine, the EJCR plots were analyzed and adequate security and precision can be established for the determination of OFLO, ENRO and NOR in serum (Fig. 7). The repeatability was analyzed by injection of 10 individual fortified serum samples. For each fluoroquinolone 2 ng were injected. The R.S.D. values are higher than those obtained in the urine analysis (Table 5).

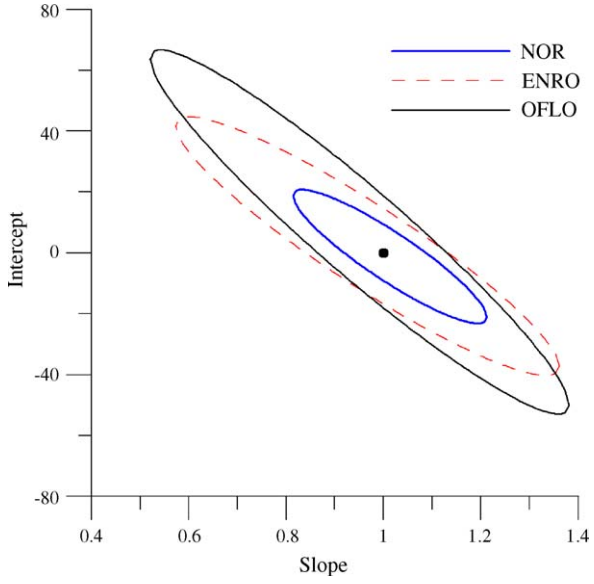

Fig. 5. Elliptical joint confidence regions for the slope and intercept, corresponding to regression of predicted vs. actual concentrations of OFLO, ENRO and NOR, and applying the proposed chromatographic method in the analysis of urine samples.

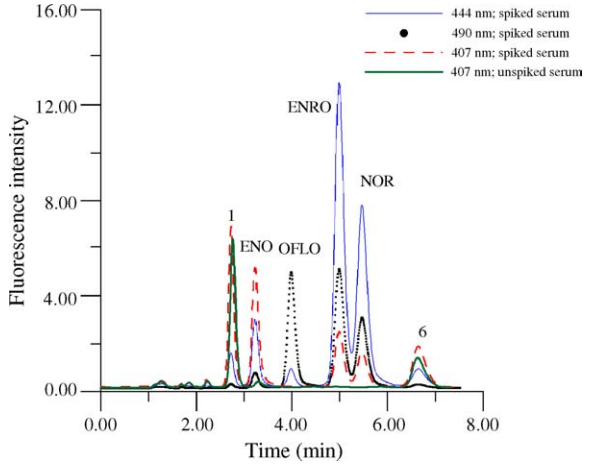

Fig. 6. Chromatograms of (- - - -) unspiked serum sample exciting at 277 nm and emission wavelength at 407 nm, and (—) spiked serum samples containing ENO (3 μg mL^{-1}), OFLO (0.16 μg mL^{-1}), ENRO (0.16 μg mL^{-1}) and NOR (0.16 μg mL^{-1}), exciting at 277 nm, and the multiemission fixed at 407, 444 and 490 nm (peaks 1 and 6 correspond to two unknown components in the serum matrix).

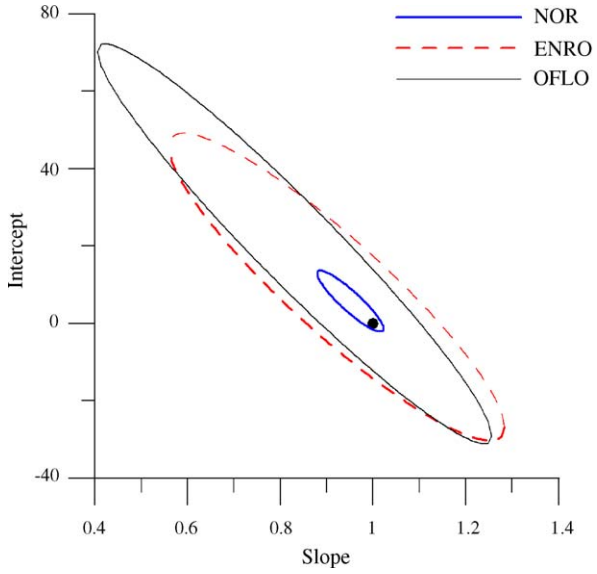

Fig. 7. Elliptical joint confidence regions for the slope and intercept, corresponding to regression of predicted vs. actual concentrations of OFLO, ENRO and NOR and applying the proposed chromatographic method in the analysis of serum samples.

4. Conclusions

The proposed LC method is simple, and a short time, less than 8 min, is necessary for compounds elution. An acceptable chromatographic resolution is obtained for the four analytes studied. The method can be applied to the analysis of NOR, OFLO and ENRO, in urine and serum samples, without pre-treatment, because only dilution is necessary. In the proposed chromatographic conditions, ENO cannot be simultaneously analyzed in a single run, in urine and serum, because is showing a low sensitivity in comparison with NOR, ENRO and OFLO. ENO shows a high resolution and adequate chromatographic parameters, to be analyzed in other type of sample, as pharmaceutical samples. Interference of the urine and serum matrix was analyzed by using the standard addition methodology. The correlation among the two applied calibration methods (external standard and standard addition methods) confirms the lack of the interference of the urine and serum matrix. Adequate recovery values from the urine and serum samples are obtained.

Acknowledgements

The authors are grateful to the MEC of Spain (Project CTQ2004-02142, co financed by Fondos Feder) for financial support of this work. David González Gómez, is grateful to the Consejería de Educación, Ciencia y Tecnología de la Junta de Extremadura for a fellowship (DOE 21/06/01).

References

[1] L.C. Jackson, L.A. Machado, M.L. Hamilton, Acta Med. 8 (1998) 58.
[2] D. Currie, L. Lynas, D.G. Kennedy, W.J. McCaughey, Food Addit. Contam. 15 (1997) 651.
[3] P.J. Ihrke, M.G. Papich, T.C. Demanuelle, Vet. Dermatol. 10 (1999) 193.
[4] D.K. Chen, A. McGeer, J.C. De Azavedo, D.E. Low, N. Engl. J. Med. 341 (1999) 233.
[5] J.S. Knapp, K.K. Fox, D.L. Tress, W.L. Whittington, Emerg. Infect. Dis. 3 (1997) 33.
[6] Food Chemical News, vol. 37, CRC Press, Washington, DC, 1996, p. 21.
[7] G. Carlucci, J. Chromatogr. A 812 (1998) 343.
[8] M.T. Maya, N.J. Goncalves, N.B. Silva, J.A. Morais, J. Chromatogr. B 755 (2001) 305.
[9] A. Zotou, N. Miltiadou, J. Pharm. Biomed. Anal. 28 (2002) 559.
[10] V.F. Samanidou, C.E. Demetriou, I.N. Papadoyannis, Anal. Bioanal. Chem. 375 (2003) 623.
[11] H.A. Nguyen, J. Grellet, B.B. Ba, C. Quentin, M.C. Saux, J. Chromatogr. B. 810 (2004) 77.
[12] O. Ballesteros, I. Toro, V. Saenz-Nebot, A. Navalon, J.L. Vilches, J. Barbosa, J. Chromatogr. B. 798 (2003) 137.
[13] M. Ferdig, A. Kaleta, T.D.T. Vo, W. Buchberger, J. Chromatogr. A. 1047 (2004) 305.
[14] B.K. Levine, Anal. Chem. 70 (1998) 209R.
[15] A. Muñoz de la Peña, A. Espinosa-Mansilla, D. González Gómez, A. Olivieri, H. Goicoechea, Anal. Chem. 75 (2003) 2640.
[16] A. Espinosa-Mansilla, A. Muñoz de la Peña, F. Salinas, D. González Gómez, Talanta 62 (2004) 853.
[17] G.L. Long, J.D. Winefordner, Anal. Chem. 55 (1983) 712.
[18] C.A. Clayton, J.W. Hines, P.D. Elkins, Anal. Chem. 59 (1987) 2506.
[19] L. Cuadros Rodríguez, A.M. García Campaña, C. Jiménez Linares, M. Román Ceba, Anal. Lett. 28 (1993) 1243.
[20] K.A. Brownlee, Statistical Theory and Methodology in Science and Engineering, Wiley, New York, 1965, p. 362.