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Determination of the chemotherapeutic quinolonic and cinolonic derivatives in urine by high-performance liquid chromatography with ultraviolet and fluorescence detection in series[1]

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Abstract

An HPLC method with ultraviolet and fluorimetric detection has been established for the separation and determination of six quinolonic and cinolonic antibiotics. A Nova-Pak C_{18} column (150×3.9 mm) and a Waters 486 UV and a Waters 470 fluorescence detector have been used. The influence of variables such as mobile-phase composition and flow-rate, has been studied. An acetonitrile–aqueous solution of oxalic acid 4×10^{-4} *M* (28:72, v/v) has been selected as optimum. The wavelength for the photometric detection of the six antibiotics was 265 nm. For the fluorimetric detection two pairs of excitation/emission wavelengths, 260/360 or 270/440 nm, were selected for the determination of nalidixic acid, 7-hydroxymethylnalidixic acid and oxolinic acid, and for the determination of pipemidic acid and cinoxacin, respectively. The analytical parameters and detection and quantification limits of the method have been determined. The proposed method has been applied for the determination of the six compounds in urine, applying different procedures depending on their concentration, the results being very acceptable. © 1997 Elsevier Science B.V.

Keywords: Quinolonic acids; Cinoxacin; Antibiotics

1. Introduction

Nalidixic (NA), oxolinic (OXO), piromidic (PIRO) and pipemidic (PIPE) acids are synthetic chemotherapeutic agents, derivatives of quinolonic acid, with very similar structures. 7-Hydroxymethylnalidixic acid (OHNA) is the major metabolite of nalidixic acid in man and animals. McChesney et al. [1] found that between 80 and 94% of the total nalidixic acid excreted is eliminated as this metabolite. Cinoxacin is a derivative of cinoline and is a chemotherapeutic agent with a structure similar to that of the other compounds.

All of these are antibacterial agents and have been extensively used in the treatment of Gram-negative urinary tract infections [2], and have also been widely used for the prevention and treatment of infectious diseases in farmed fish.

Almost all studies on their mode of action have been performed with nalidixic acid, and the results point to their influence on DNA replication in

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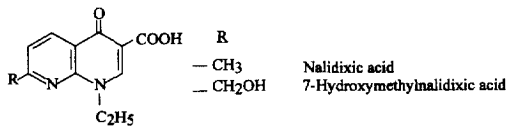
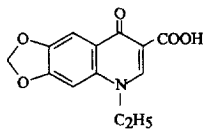
sensitive microorganisms, urine being their major way of elimination [3].

The determination of these compounds in serum and plasma usually involves HPLC methods with UV detection. Sorel et al. [4] proposed a chromatographic method for the determination of OHNA in plasma after extraction with $CHCl_3$. Cuisinaud et al. [5] determined NA and its two major metabolites in plasma and in urine, and other methods are described for the determination of NA [6,7] or PIPE [8–10]. Even when HPLC methods for the analysis of some of the cited compounds in other types of samples appear in the literature, we have not found a determination method for the analysis of all of them.

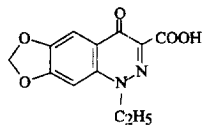
In the present study, a HPLC method is reported for the simultaneous determination of the six quinolonic and cinolonic derivatives in urine samples, using a combination of photometric and fluorimetric detection.



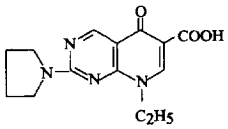
Nalidixic acid
7-Hydroxymethylnalidixic acid



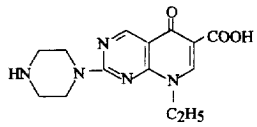
Oxolinic acid



Cinoxacin



Piromidic acid



Pipemidic acid

2. Experimental

2.1. Apparatus and chromatographic conditions

The studies carried out were performed on a Waters 600E high-performance liquid chromatograph equipped with a Waters 610 pump, a Waters 486 UV detector and a Waters 470 fluorescence detector (Waters Millipore, Milford, MA, USA). The system was equipped with a six-way injection valve (Rheodyne), containing a 20-μl loop, and an analytical column Nova-Pak C_{18} (150×3.9 mm) (Waters Millipore). Data acquisition and data analysis were performed with the Maxima 825 software package, Version 3, supplied by Waters.

The eluent was prepared by mixing an aqueous solution of 4×10^{-4} *M* oxalic acid with acetonitrile in a 72:28 proportion. The eluent was filtered through a 0.45-μm cellulose acetate filter and degassed before used. The flow-rate was 2.0 ml/min.

Before injection, samples were filtered through a Millipore Swineex syringe adaptor (Millipore Bedford, MA, USA), containing 0.45-μm regenerated cellulose membrane filters. Photometric detection was performed at 265 nm, and fluorimetric detection at $\lambda_{ex}/\lambda_{em}$, 260/360 or 270/440 nm.

2.2. Chemicals and reagents

Nalidixic acid (NA), oxolinic acid (OXO), pipemidic acid (PIPE), piromidic acid (PIRO) and cinoxacin (CINOX) were obtained from Sigma (St. Louis, MO, USA) and used as received. 7-Hydroxymethylnalidixic acid (OHNA) was obtained from Sanofi Wintrop (Sanofi Wintrop S.A., Barcelona, Spain). HPLC-grade water was obtained from Water Pro PS (Labconco, Kansas City, MO, USA). Acetonitrile for chromatography and oxalic acid from Merck were also used. All other chemicals were of analytical reagent grade.

2.3. Procedure for the analysis of urine samples

2.3.1. Photometric determination

Aliquots of 1 ml of urine containing more than 100 μg ml^{-1} of these antibiotics, were diluted to 25 ml with the mobile phase, and aliquots of 20 μl were injected in the chromatographic system. The signal was measured at 265 nm.

2.3.2. Fluorimetric determination

Aliquots of 1 ml of urine, containing more than 0.2 μg ml^{-1} of each antibiotic, were placed in a

separating funnel and diluted to 25 ml with deionized water. The pH was adjusted to 2.5–3. This solution was extracted with 25 ml of chloroform. After the phases were allowed to separate, the organic phase was evaporated to dryness. The residue was dissolved in 3 ml of acetonitrile, transferred into a 10-ml calibrated flask (or greater volume if convenient), and diluted to volume with water. Aliquots of 20 μl were injected in the chromatographic system. For the analysis, the fluorescence was measured at 360 nm, exciting at 260 nm, and at 440 nm, exciting at 270 nm.

3. Results and discussion

3.1. Optimization of the mobile phase

All of these antibiotics present a maximum of absorption near to 265 nm, as is shown in Fig. 1, and this wavelength was selected for the realization of this study. First, the variation of the retention time of these peaks with the mobile-phase composition, and the flow-rate of the mobile phase was studied. Different mobile phases, methanol–oxalic acid and acetonitrile–oxalic acid were tested, since this acid has been used in other cases to obtain an adequate pH value of the mobile phase in the separation of some of the compounds studied. The capacity factors or resolution values obtained were better when acetonitrile was used as organic modifier, and this solvent was selected as optimum. The amount of acetonitrile was varied between 23 and 45%, and the retention time of the peaks increases when decreasing the amount of acetonitrile in the mobile phase. The concentration of oxalic acid was varied between 4×10^{-4} and 7×10^{-4} *M*. In general, the retention time of the analytes slightly increases by increasing the concentration of the oxalic acid of the mobile phase, except in the case of pipemidic acid. The retention time of this analyte increases by decreasing the concentration of oxalic acid. From these studies, the mobile phase selected as optimum was acetonitrile–aqueous oxalic acid 4×10^{-4} *M* (28:72, v/v).

The flow-rate was varied between 1 and 2.5 ml/min. It was found that the flow-rate mainly affects the performance of the system for nalidixic and piromidic acids. On the other hand, in all cases the resolution was to the baseline. A flow-rate of 2 ml/min was selected and all the compounds were eluted in less than 10 min, as shown in Fig. 2. Under these conditions, the retention time and capacity factor values were: OHNA 1.13 min and 1.06, CINOX 1.64 min and 1.98, OXO 2.15 min and 2.91,

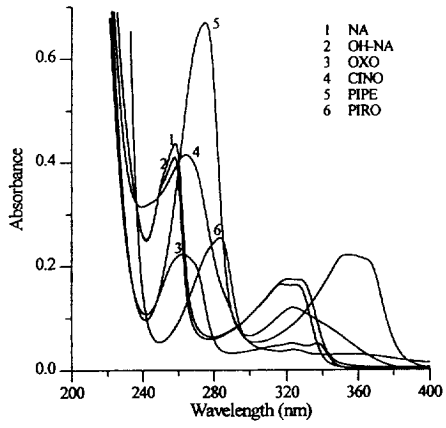


Fig. 1. Absorption spectra of the chemotherapeutic quinolonic and cinolonic derivatives. Concentration of each is 20 μg ml^{-1}, pH 4.5.

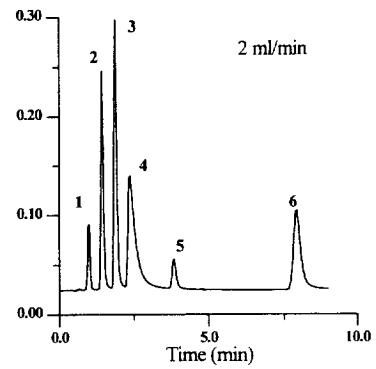


Fig. 2. Chromatogram of a sample of the six antibiotics; mobile phase, acetonitrile–aqueous oxalic acid 4×10^{-4} *M* (28:72, v/v); flow-rate of 2 ml/min; photometric detection. Peaks: 1, OHNA; 2, CINOX; 3, OXO; 4, PIPE; 5, NA; 6, PIRO.

PIPE 2.97 min and 4.39, NA 4.24 min and 6.71 and PIRO 8.51 min and 14.45, respectively.

3.2. Selection of the instrumental variables for fluorimetric detection

All the compounds studies show native fluorescence under the conditions used, but they present different wavelengths for their optimum detection. For the selection of a wavelength that allows us to determine the majority of the compounds with the highest sensitivity, the total luminescence spectrum of each of these compounds was registered in the form of an emission/excitation matrix (EEM). The

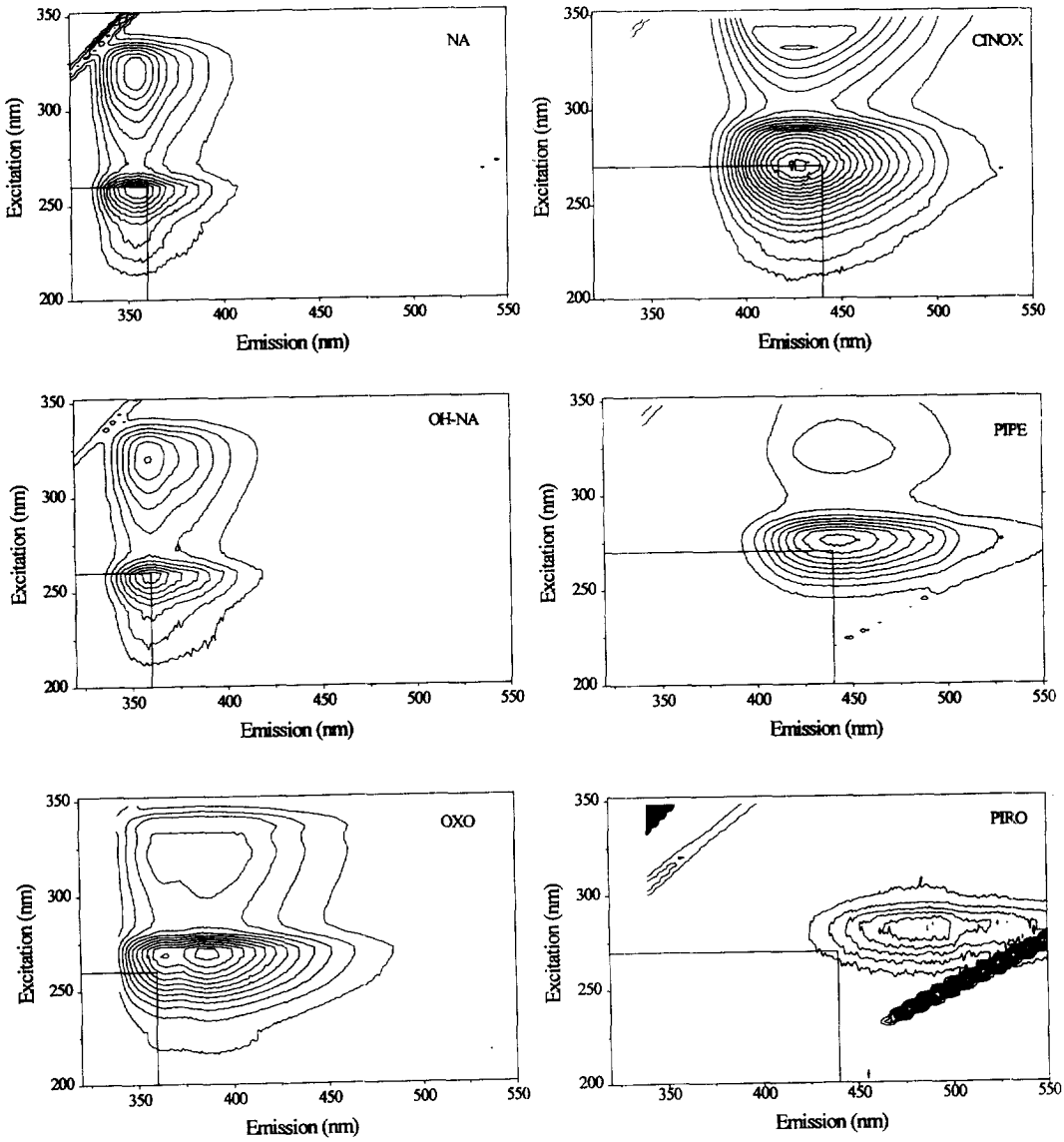


Fig. 3. Contour plots of the total fluorescence spectra of NA, OHNA, OXO, CINOX, PIPE and PIRO in acetonitrile–aqueous solution of oxalic acid 4×10^{-4} *M* (28:72, v/v). The selected pairs of excitation/emission wavelengths selected for obtaining the chromatograms are shown by the solid lines slicing the data matrix.

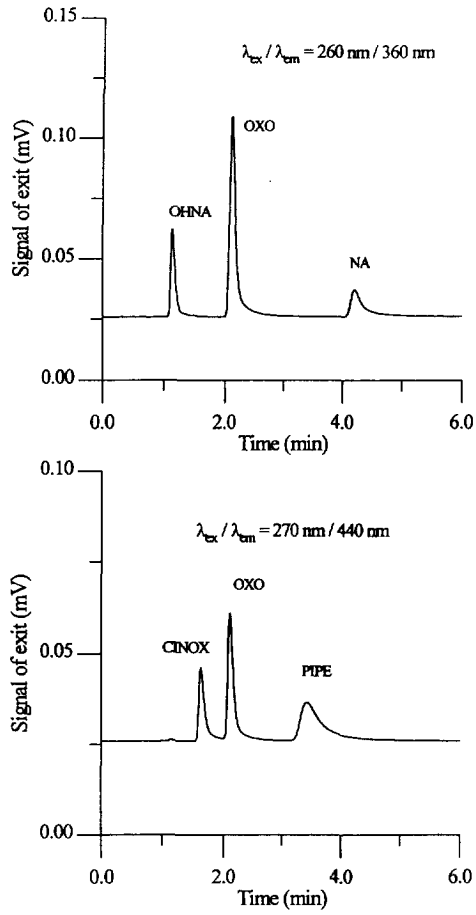


Fig. 4. Chromatogram obtained for the six antibiotics with fluorimetric detection at 260/360 nm and at 270/440nm. [OHNA]=0.1 μg ml^{-1}; [OXO]=0.1 μg ml^{-1}; [NA]=0.2 μg ml^{-1}; [CINOX]=0.05 μg ml^{-1}; [PIPE]=0.1 μg ml^{-1}.

emission spectra were scanned between 240 and 550 nm, at increments of the excitation wavelength of 4 nm, between 200 and 350 nm, and displayed as contour plots. In Fig. 3, the contour plots of all the antibiotics are presented. It can be seen that the selection of only one excitation/emission wavelength pair for the determination of all of the compounds is not possible. Therefor, two different pairs of excitation/emission wavelengths were selected. The first pair was 360 nm, exciting at 260 nm, for the detection of nalidixic, 7- hydroxymethylnalidixic and oxolinic acids, and the other was 440 nm, exciting at 270 nm, for the detection of pipemidic acid, cinoxacin and, with minor sensitivity, oxolinic acid. Piromidic acid was not detected by fluorescence, as it does not present a suitable signal, the sensitivity of the fluorimetric detection being similar to that obtained by photometric detection. The chromatograms obtained under these conditions are shown in Fig. 4.

3.3. Analytical parameters

3.3.1. Photometric detection

Under the selected conditions, calibration graphs were obtained by preparing samples in triplicate with increasing concentrations of each analyte. Each sample was injected three times in the chromatographic system, and the mean of the obtained area or height values was plotted against concentration. The study was performed with concentrations of each antibiotic between 0.25 and 40 μg ml^{-1}. The results obtained are summarized in Table 1, which also

Table 1
Analytical parameters for the chemotherapeutic quinolonic and cinolonic derivatives with photometric detection (λ=265 nm)

Compound	Signal	Correlation coefficient (R)	R.S.D. (%)	Analytical sensitivity[a]	LOD[b] (μg ml^{-1})
OHNA	Height	0.9956	1.45 (7.5 μg ml^{-1})	2.02	1.42
	Area	0.9961	2.65 (7.5 μg ml^{-1})	1.97	1.25
CINOX	Height	0.9977	2.06 (3.75 μg ml^{-1})	3.98	0.60
	Area	0.9968	4.71 (3.75 μg ml^{-1})	3.39	0.70
OXO	Height	0.9974	1.90 (7.5 μg ml^{-1})	1.90	1.26
	Area	0.9981	2.27 (7.5 μg ml^{-1})	2.17	1.09
PIPE	Height	0.9960	2.57 (7.5 μg ml^{-1})	1.64	1.42
	Area	0.9974	2.18 (7.5 μg ml^{-1})	2.03	1.15
NA	Height	0.9971	1.71 (15 μg ml^{-1})	0.89	2.74
	Area	0.9963	2.14 (15 μg ml^{-1})	0.78	3.09
PIRO	Height	0.9976	2.77 (7.5 μg ml^{-1})	1.98	1.28
	Area	0.9985	2.22 (7.5 μg ml^{-1})	2.48	1.02

[a] Analytical sensitivity: slope of calibration curve/residual mean [12].
[b] Calculated by Clayton's method ($\alpha=\beta=0.05$) [11].

Table 2
Analytical parameters for the chemotherapeutic quinolonic and cinolonic derivatives with fluorimetric detection at the two pairs of excitation/emission wavelengths selected

Compound	Signal	Correlation coefficient (R)	R.S.D. (%)	Analytical sensitivity[a]	LOD[b] (ng ml^{-1})
OHNA[c]	Height	0.9983	4.62 (40 ng ml^{-1})	0.40	8.77
	Area	0.9988	6.52 (40 ng ml^{-1})	0.15	10.4
OXO[c]	Height	0.9983	3.14 (40 ng ml^{-1})	0.24	9.47
	Area	0.9978	4.08 (40 ng ml^{-1})	0.21	10.8
NA[c]	Height	0.9985	2.08 (80 ng ml^{-1})	0.12	19.7
	Area	0.9982	4.05 (80 ng ml^{-1})	0.11	21.6
CINOX[d]	Height	0.9978	8.17 (22 ng ml^{-1})	1.05	2.43
	Area	0.9984	7.62 (22 ng ml^{-1})	1.23	2.05
OXO[d]	Height	0.9978	2.96 (43.7 ng ml^{-1})	0.51	4.98
	Area	0.9972	2.83 (43.7 ng ml^{-1})	0.45	5.63
PIPE[d]	Height	0.9983	5.73 (43.7 ng ml^{-1})	0.59	4.24
	Area	0.9990	6.07 (43.7 ng ml^{-1})	0.78	3.26

[a] Analytical sensitivity: slope of calibration curve/residual mean [12].
[b] Calculated by Clayton's method ($\alpha=\beta=0.05$) [11].
[c] $\lambda_{ex}=260$ nm, $\lambda_{em}=360$ nm.
[d] $\lambda_{ex}=270$ nm, $\lambda_{em}=440$ nm.

includes the detection limit values calculated from the standard deviation values of slope and origin intercept, and choosing a false-positive and a false-negative probability value of 0.05 [11].

3.3.2. Fluorimetric detection

This procedure is similar to that described for photometric detection, but the concentration range is, in general, between 5 and 450 ng ml^{-1}, depending on the antibiotic, and each sample was injected six times in the chromatograph. In three of the cases, the detection was carried out at 360 nm, exciting at 260 nm, and in the others at 440 nm, exciting at 270 nm. The correlation coefficient and the detection limits at the two pairs of wavelengths are summarized in Table 2.

3.4. Applications

The proposed method has been applied to the determination of these antibiotics in spiked human urine samples. Fig. 5 summarizes the different steps followed in the treatment of the urine samples. First, the determination of the antibiotics in urine samples

Table 3
Results obtained in the analysis of chemotherapeutic quinolonic and cinolonic derivatives in spiked urine samples with photometric detection ($\lambda=265$ nm)

Compound	Added (μg ml^{-1})	Recovery (%)[a]	
		Height	Area
OHNA	124	101.9	104.8
	186	120.1	104.3
	248	117.1	95.2
CINOX	125	98.4	92.8
	195	102.2	94.7
	260	102.8	96.3
OXO	123	95.1	99.2
	210	112.1	97.9
	280	115.6	98.7
PIPE	123	109.8	97.8
	206	117.6	101.1
	274	121.8	101.6
NA	124	89.8	96.5
	320	105.9	99.8
	426	106.5	99.7
PIRO	123	94.9	95.7
	206	98.5	96.6
	274	101.0	95.7

[a] Mean recovery of three separate determinations.

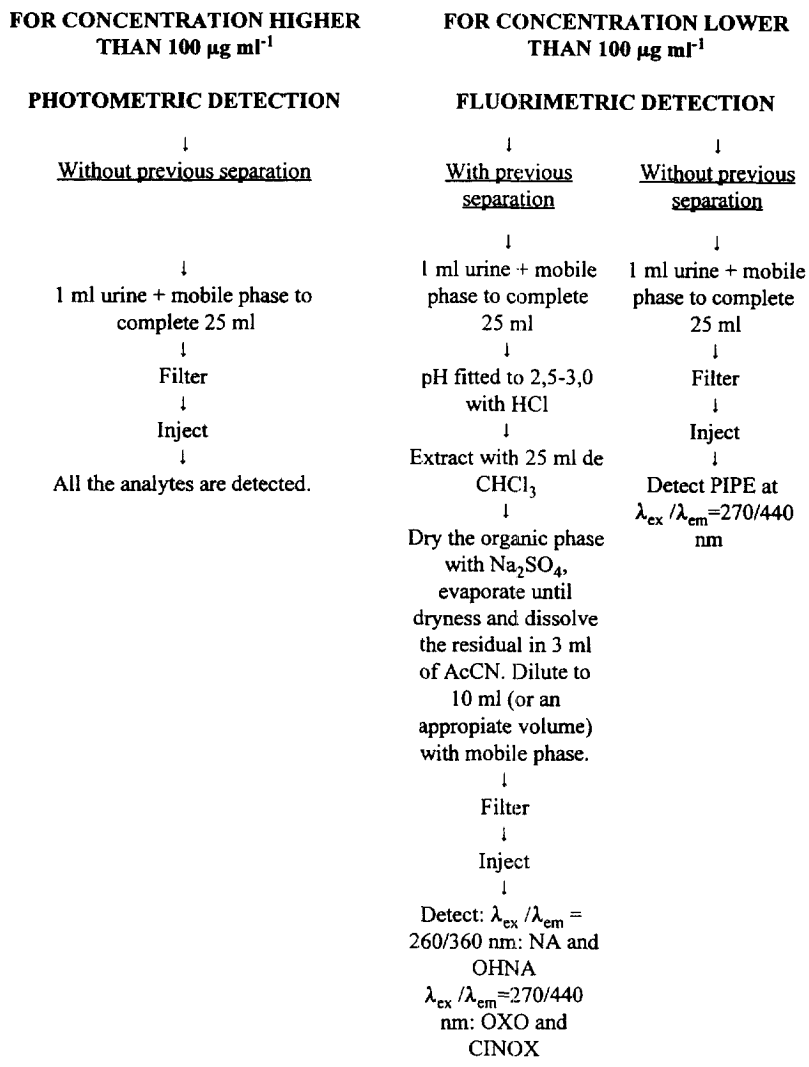


Fig. 5. Different procedures for the treatment of the urine samples.

without pretreatment was tried, taking 1 ml of urine, and diluting to 25 ml with the mobile phase. Using photometric detection, the six antibiotics could be determined at concentration levels of 100 μg ml^{-1} or higher with good results, which are summarized in Table 3. When fluorimetric detection is used, measuring at the two pairs of wavelength mentioned above, only NA and PIPE could be determined, and no satisfactorily results were obtained for the other analytes, due to the signal of the urine matrix. With the object of determining all of these antibiotics fluorimetrically and in lower concentrations, two different pretreatment procedures were assayed with spiked urine samples. The first consists of a solid–liquid extraction, using a Sep-Pak Plus C_{18} (Waters) cartridge. The Sep-Pak Plus was previously conditioned with 5 ml of acetonitrile and with 5 ml of water. All the analytes are retained in the cartridge, except the pipemidic acid, which is only partially retained. An attempt to improve the retention of this analyte was made varying the pH between 2 and 5, but better results were not obtained. The second method consists of a liquid–liquid extraction of urine, at pH 3, with chloroform, allowing the phases

to separate, evaporating the organic phase to dryness, and following the above-described procedure. In this procedure, the pipemidic acid is not extracted at any pH value. Due to the problem with the pipemidic acid, and as this compound can be determined at a low concentration level in urine using a simple dilution without any additional pretreatment, and using fluorimetric detection, this procedure was chosen for this analyte. Fig. 6 presents the chromatograms, obtained at the two pairs of wavelengths selected, for a urine sample without any pretreatment, in comparison with those obtained when a solid–liquid extraction or a liquid–liquid extraction was performed. It can be seen that the urine matrix is better cleaned with a liquid–liquid extraction. Fig. 7 presents the chromatogram obtained with a spiked urine sample treated with a liquid–liquid extraction, together with the chromatogram of a standard of the five antibiotics. It can be observed that the urine matrix does not interfere with the peaks that are measured, except in the case of the detection of

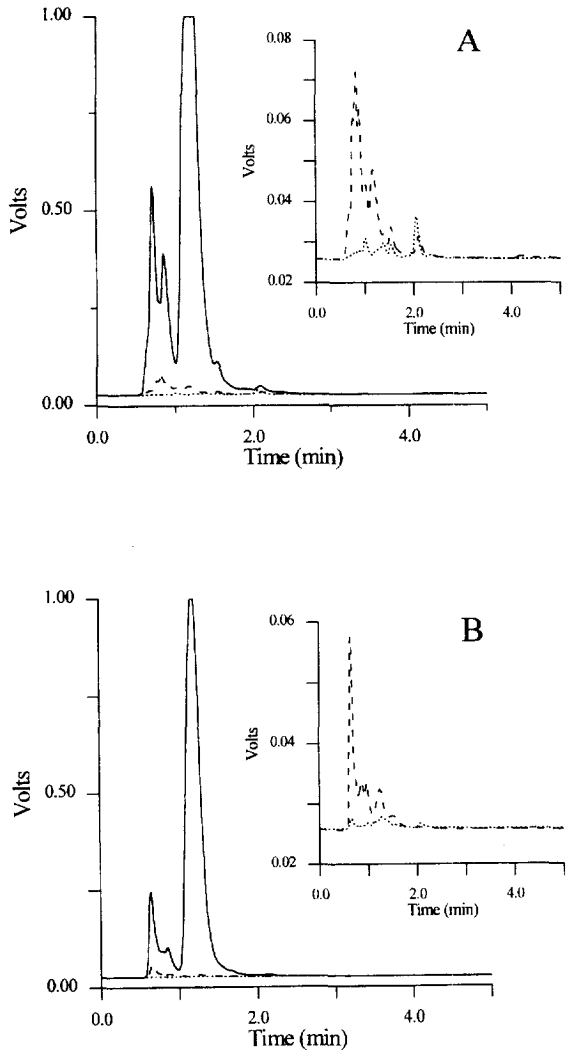


Fig. 6. Chromatogram of a urine sample (fluorimetric detection): (A) at 360 nm, exciting at 260 nm; and (B) at 440 nm, exciting at 270 nm. Urine sample without pretreatment (——) ; urine sample with a previous solid–liquid extraction (- - -) ; and urine sample with a previous liquid–liquid extraction (. . .).

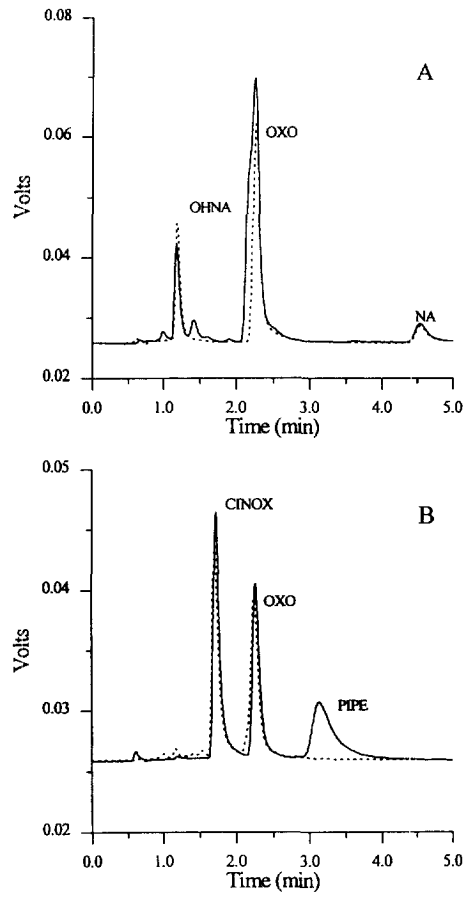


Fig. 7. Chromatogram of an urine sample spiked with the antibiotics and subjected to a liquid-liquid extraction (. . .); chromatogram of a standard of the antibiotics (fluorimetric detection) (——). (A) At 360 nm, exciting at 260 nm; and (B) at 440 nm, exciting at 270 nm.

oxolinic acid at 260/360 nm. Because of this, this analyte was determined at 270/440 nm.

The obtained results were excellent as shown in Table 4. Concentrations of the antibiotics in the low ppm level can be determined, with a great simplicity and speed.

Table 4
Results obtained in the analysis of chemotherapeutic quinolonic and cinolonic derivatives in spiked urine samples with fluorescence detection following the procedures described in Section 3.4

Compound	Added ($\mu g\ ml^{-1}$)	Recovery (%)[a]	
		Height	Area
OHNA[b]	0.250	104.5	103.8
	0.375	92.05	88.7
	0.624	77.6	89.8
	0.749	92.4	94.1
	1.00	98.9	105.1
NA[b]	0.250	100.2	104.8
	0.500	104.6	111.6
	0.625	99.9	101.4
	0.750	110.9	104.8
	1.000	108.9	110.7
CINOX[c]	0.250	82.8	92.3
	0.374	101.3	100.5
	0.607	87.9	92.6
	0.748	92.4	94.1
	0.997	93.1	93.5
OXO[c]	0.375	118.1	126.2
	0.500	99.7	110.5
	0.624	104.8	113.9
	0.722	96.0	107.7
	0.962	107.4	120.0
PIPE[c]	0.590	97.5	86.4
	1.190	104.4	84.0
	2.380	105.5	88.0

[a] % Mean recovery of three separate determinations.
[b] λ_{ex}=260 nm, λ_{em}=360 nm.
[c] λ_{ex}=270 nm, λ_{em}=440 nm.

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