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Determination of piromidic acid residues in trout muscle tissue and in urine by liquid chromatography with post-column modification of pH and fluorimetric detection

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Abstract

A rapid high-performance liquid chromatographic method has been developed to determine piromidic acid in trout muscle tissue and in urine, in the presence of nalidixic, 7-hydroxymethylnalidixic, oxolinic and pipemidic acids and cinoxacin. A Nova-Pak C_{18} column was used with acetonitrile–$4 \cdot 10^{-4}$ *M* oxalic acid (40:60, v/v) as the mobile phase. A post-column change of pH was made with NaOH. Fluorimetric detection at 456 nm (λ_{ex} 275 nm) was used. The instrumental detection limit was 5.91 ng/ml, based on height of peak. Pretreatment of the urine samples was not necessary and fish samples were extracted with sodium hydroxide solutions and cleaned by means of an extraction with chloroform. Detection limit was 147 ng/ml for urine and 5.91 ng/g for trout muscle. Good separation without interference from any other components was obtained. Recovery was better than 87% in urine and better than 72% in trout muscle tissue.

Keywords: Piromidic acid

1. Introduction

Piromidic acid (PIRO) (8-ethyl-5,8-dihydro-5-oxo-2-(1-pyrrolidinyl)pyrido[2,3-d]pyrimidine-6-carboxylic acid) is a synthetic antimicrobial drug that was first proposed by Shimizu et al. [1]. This compound is extensively used in the treatment of Gram-negative urinary tract infections [2] and in fisheries, particularly in fish breeding. Concern about residues of this antibiotic in edible fish has created a need for its determination.

The usual methods for the determination of piromidic acid are HPLC with UV detection. Kasuga et al. [3,4] proposed two HPLC methods for the determination of piromidic acid in fish and in the presence of nalidixic and oxolinic acids, at 335 and 330 nm. The detection limit of the method was 0.1 μg/ml. Other authors investigated the determination of piromidic acid in the presence of nalidixic and oxolinic acids in fishes with UV detection [5–7].

Other methods combined photometric with fluorimetric detection. Horie et al. [8], proposed an HPLC method in which they determine piromidic acid at 280 nm and nalidixic and oxolinic acid with fluorimetric detection. Munns et al. [9] proposed an HPLC method for the determination of flumequine and nalidixic, oxolinic and piromidic acids in catfish using UV detection, at 280 nm, for piromidic acid

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and fluorimetric detection for the other compounds. Pfenning et al. [10] applied the previous method for the determination of quinolones in salmon and recoveries for piromidic acid were between 75 and 91%. Horie and co-workers [11,12], analyzed piromidic acid in fish, pork, chicken and leaves, in the presence of other quinolonic antibacterials, with photometric detection for its determination and fluorimetric detection for the other compounds.

Horie et al. [13] used thermospray liquid chromatography–mass spectrometry to confirm residues of piromidic, oxolinic and nalidixic acids in fish. Gas chromatography has been used for the determination of these quinolones after conversion in their methyl esters [14]. Takatsumki [15] used GC–MS for the determination of nalidixic, oxolinic and piromidic acids in fish. The recoveries of piromidic acid were greater than 69.6%.

Sun and Chen [16] optimized the separation of 14 quinolonic antibacterials, including piromidic acid, by capillary electrophoresis using the overlapping resolution mapping scheme in a time of 8 min. Álvarez et al. [17] studied the nature of several quinolone–metal complexes by electrospray ionization and laser desorption/ion-molecule.

Piromidic acid forms a complex with iron (III) in the presence of *o*-hydroxyquinolphthalein and, in these conditions, the antibiotic has been determined, at 615 nm, in pharmaceuticals and in calf serum [18].

We have not found in the literature any reference to either the determination of piromidic acid by fluorescence or the fluorescent characteristics of this compound.

In this paper, we describe the luminescent characteristics of piromidic acid. Also, a HPLC method is reported for its determination in the presence of five other quinolonic and cinolonic antibiotics, in urine and in fish, using fluorimetric detection.

2. Experimental

2.1. Apparatus

Fluorescence measurements were made on a SLM Aminco-Bowman Series 2 luminescence Spectrometer (SLM Instruments, Rochester, NY, USA), equipped with a 150 W continuous Xenon lamp, interfaced with a GPIB (IEE-488) card and driver with a personal computer 386 microcomputer. Data acquisition and data analysis were performed by the AB2 software, version 1.40, running under OS/2 2.0. The excitation and emission slits were maintained at 4 and 8 nm, respectively. The scan rate of the monochromators was 10 nm/s. All measurements were performed in a 10-mm quartz cell and at 10 ± 0.1°C by use of a thermostatically controlled cell holder and a Selecta Model 382 thermostatically controlled water-bath.

HPLC was carried out in a Waters 600E high-performance liquid chromatograph, equipped with a Waters 610E pump, a Waters 486 UV-detector and a Waters 470 fluorescence detector (Waters Millipore, 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 using the MAXIMA 825 software package, version 3, supplied by Waters.

The eluent was prepared by mixing an aqueous solution of $4\cdot10^{-4}$ *M* oxalic acid with acetonitrile in a 60:40 (v/v) 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.

After the column, an additional pump, Hurst A-30-SW-2, supplied by Waters, with a micrometric screw to vary the flow between 0.2 and 1.45 ml/min, drives a $1.5\cdot10^{-2}$ *M* sodium hydroxide solution to be mixed with the column eluent, through a T-connection. Before injection, samples were filtered through a filter syringe adaptor, containing 0.45-μm nylon membrane filters (Lida, WI, USA). Fluorimetric detection was performed at 456 nm (λ_{ex} 275 nm).

2.2. Reagents and chemicals

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, Barcelona, Spain). A stock solution of piromidic acid was prepared in a 100-ml volumetric flask by dissolving it in 6 ml of

dimethylformamide (Panreac Química, Barcelona, Spain) and diluting to the mark with acetonitrile. Stock solutions of the other antibiotics, were prepared in acetonitrile. In all cases, solutions of lower concentrations were prepared by appropriate dilution of the stock solutions with mobile phase or chloroform. HPLC grade water was obtained from Water Pro™ PS, Labconco system (Kansas City, MO, USA). Acetonitrile for chromatography and oxalic acid from Merck (Darmstadt, Germany) were also used. All other chemicals were of analytical reagent grade.

2.3. General procedure for the fluorimetric determination of piromidic acid

Aliquots of the PIRO stock solution were transferred to a 25.0 ml volumetric flask, for a final concentration between 0.85 and 7.65 μg/ml. Then, 1 ml of 0.5 *M* NH_3–NH_4Cl (pH 11) buffer solution and water to the final volume, were added. Fluorescence was measured at λ_{em} 456 nm, λ_{ex} 275 nm.

2.4. General procedure for the determination of PIRO by HPLC

Aliquots of the PIRO solution, containing above 50 ng, were placed in a 10.0-ml volumetric flask and diluted with the mobile phase to the final volume. A 20-μl volume was injected into the chromatographic system.

2.5. Procedure for the analysis of urine samples

Urine (1-ml aliquots) containing more than 125 ng/ml of the antibiotic were diluted in a 25-ml volumetric flask with mobile phase, and 20-μl volumes were injected into the chromatographic system. The signal was measured at 456 nm, λ_{ex} 275 nm.

2.6. Procedure for the analysis of trout muscle tissue

A 2-g amount of the sample previously crushed with an electric mixer, was weighted and manually homogenized with 10 ml of 0.1 *M* NaOH in 50-ml centrifuge tube for 2 min. It was centrifuged at 4100 rpg for 5 min and the supernatant decanted into a separatory funnel. The extraction was repeated with 10 ml of NaOH and the extracts combined in the separatory funnel. Chloroacetic acid (1 *M*) was added to pH 3 and it was extracted with two portions of 25 ml of chloroform, shaken vigorously and the phases allowed to separate. The organic phases were combined and centrifuged at 4100 r.p.m. for 5 min. The organic phase was evaporated in the rotary evaporator at 50°C and dissolved in 2 ml of phase mobile. Aliquots of 20 μl were injected into the chromatographic system. The signal was measured at λ_{em} 456 nm, λ_{ex} 275 nm.

3. Results and discussion

3.1. Fluorimetric study of piromidic acid

The native fluorescence of PIRO in aqueous medium (pH <7.0) is relatively weak. The excitation and emission fluorescence spectra of PIRO are shown in Fig. 1. The excitation spectrum presents two maxima located at 275 and 335 nm. The shorter excitation wavelength has been used for the spectrofluorimetric studies, as the emission intensity obtained is higher than that achieved with the use of the longer wavelength. The wavelengths chosen were 275 and 456 nm for excitation and emission, respectively.

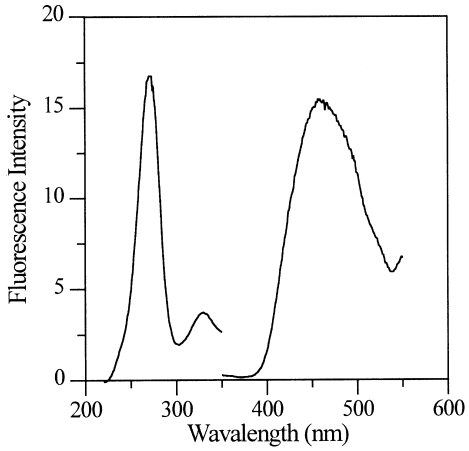

Fig. 1. Excitation (left) and emission (right) spectra of PIRO in aqueous media; [PIRO]=$1.05 \cdot 10^{-5}$ *M*.

A study of the influence of pH on the fluorescence intensity of PIRO was carried out. The pH of all solutions was varied, over the range 1.5–12.0, by the addition of trace amounts of hydrochloric acid or sodium hydroxide. The results obtained are presented in Fig. 2. The fluorescence intensity shows a maximum and constant value in the pH range of 7.5–13.0, whereas the fluorescence decreases for values of pH lower than 7, being practically negligible and constant for pH $\leq$4.5. A pH value of 11, obtained by the addition of 1.00 ml of ammonia–ammonium chloride buffer solution, was selected as optimum.

The value of the pK_a, calculated by the method of Stenström and Goldsmith [19], adapted to fluorescence measurements [20] was 6.00$\pm$0.33, and using the Wilson and Lester method [21] a value of 6.05$\pm$0.10 was found.

The dependence of the fluorescence intensity of the antibiotic on the temperature is critical. The fluorescence emission decreases as the temperature increases. A constant temperature of 10°C is recommended.

The calibration graph is linear over the range 0.8–7 μg/ml. The detection limit, calculated by Clayton et al. [22], is 0.61 μg/ml. For a series of ten measurements of a solution containing 2.5 μg/ml of PIRO, the relative standard deviation was 0.73% and the relative error was 1.0% (95% confidence level).

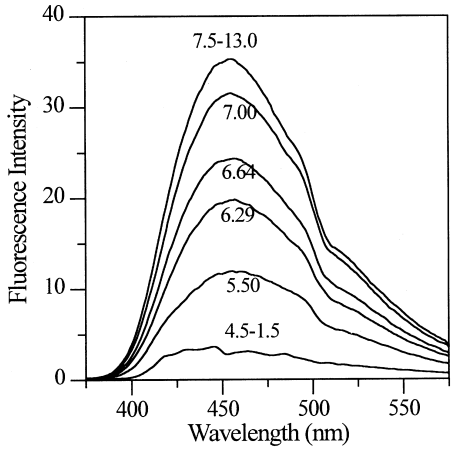

Fig. 2. Influence of the pH on the emission spectra of the PIRO solution; λ_{ex}=275 nm, λ_{em}=456 nm, [PIRO]=1.77·10^{-5} *M*.

3.2. HPLC analysis of the piromidic acid with fluorimetric detection

In a previous paper, we studied the resolution of a mixture of six quinolonic and cinolonic antibiotics (nalidixic acid, 7-hydroxymethylnalidixic acid, oxolinic acid, pipemidic acid, piromidic acid and cinoxacin) using reversed-phase chromatography, with a C_{18} column and a mobile phase of acetonitrile–oxalic acid. In the selected conditions in that paper [23], the sensitivity for piromidic acid, with fluorimetric detection, was of the same order as the sensitivity obtained with photometric detection. A better sensitivity for the fluorimetric detection is difficult to achieve, with any other conditions, as the pH of the mobile phase must be maintained near to or lower than 7, in order to ensure the stability of the stationary phase. This is why we propose now to make a post-column change of the pH with the aim of increasing the sensitivity for the determination of the piromidic acid in presence of the other five antibiotics.

3.3. Optimization of the mobile phase

In the mentioned paper, the mobile phase, selected for the separation of the six quinolonic and cinolonic antibiotics, was acetonitrile–4·10^{-4} *M* oxalic acid (28:72, v/v) and under these conditions the time for the analysis of the piromidic acid was 8.5 min. In order to shorten the time of the analysis for the piromidic acid, we examined the influence of the proportion of acetonitrile in the mobile phase, between 28% and 40%. As expected, the retention time of the piromidic acid decreases with increasing amount of acetonitrile in the mobile phase. From these studies, the mobile phase selected as optimum was acetonitrile–4·10^{-4} *M* oxalic acid (40:60, v/v). The chromatogram obtained for a mixture of the six antibiotics, under these conditions, with a flow-rate of 2 ml/min and photometric detection (λ =265 nm), is shown in Fig. 3A. Only five peaks appear because the peaks of the pipemidic and the piromidic acids overlap, as we can see in Fig. 3B. However, when the pH was varied post-column, and with fluorimetric detection, only the peak corresponding to the piromidic acid was observed, because

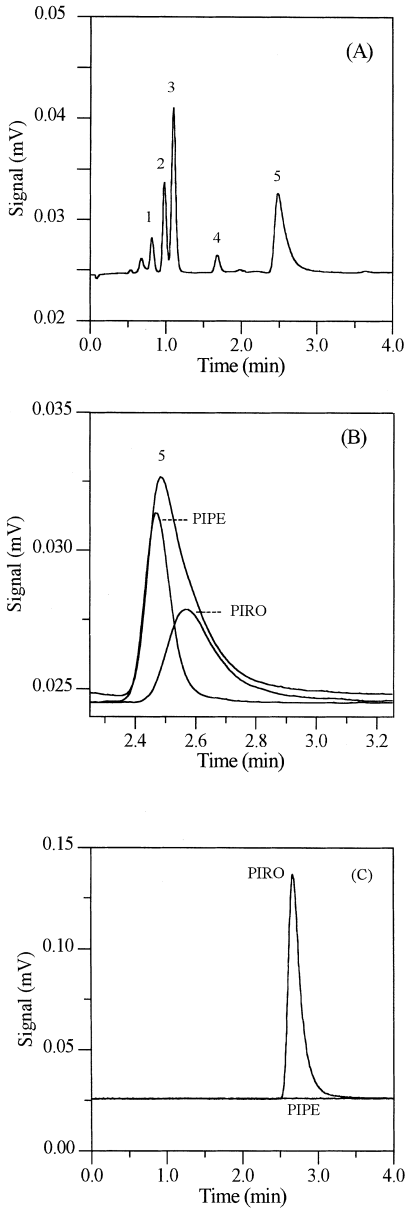

Fig. 3. Chromatogram of a mixture of the six antibiotics, using as mobile phase acetonitrile–aqueous solution of oxalic acid $4 \cdot 10^{-4}$ *M* (40:60, v/v), a flow-rate of 2 ml/min and photometric detection at 265 nm; (A) 1=7-hydroxymethylnalidixic acid; 2=cinoxacin; 3=oxolinic acid; 4=nalidixic acid; 5=pipemidic and piromidic acids; (B) Chromatogram of samples of PIPE and PIRO and a mixture of both at 265 nm; (C) Chromatogram of samples of PIPE and PIRO with fluorescence detection and after post-column change of pH with NaOH, λ_{ex}=275 nm, λ_{em}=456 nm.

pipemidic acid and the other antibiotics are not fluorescent at pH values above 7.0 (Fig. 3C).

The concentration of the NaOH solution used was varied between $5 \cdot 10^{-3}$ and $2 \cdot 10^{-2}$ *M*. The signal increases with the concentration of NaOH until a value of $1.5 \cdot 10^{-2}$ *M* NaOH, which was selected as the optimum. The flow-rate of the NaOH solution was varied between 0.56 and 1.45 ml/min with a constant flow-rate of the mobile phase of 2 ml/min. The signal decreases as the flow of the NaOH increases. A flow-rate of 0.56 ml/min was chosen as optimum. Under these conditions, the retention time and the capacity factor values for the piromidic acid were 2.55 min and 3.64, respectively. Hence, it is possible to determine the piromidic acid in a very short time, with high sensitivity, in presence of other very similar compounds.

3.4. Analytical parameters

Under the selected conditions, calibration graphs were obtained by preparing samples in triplicate with increasing concentrations. 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 realized between 5 and 100 ng/ml. The detection was carried out λ_{em} 456 nm, λ_{ex} 275 nm. The results obtained are summarized in Table 1, which also includes the detection limits 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 [22].

3.5. Applications

The proposed method has been applied to the determination of PIRO in spiked human urine and trout muscle tissue samples. In both instances, the accuracy and precision are satisfactory.

3.5.1. Urine

The proposed method has been applied to several spiked human urine samples from normal adults. It is not necessary to pretreat the urine. A 1-ml volume of urine, containing above 1.0 μg/ml of the antibiotic, was diluted to 25 ml with the mobile phase. Aliquots of 20 μl were injected into the system. Fig. 4A

Table 1
Analytical parameters for the determination of the piromidic acid by HPLC with fluorescent detection and post-column modification of pH

Analytical signal	Height	Area
Equation	H=220.3 []+443.7	A=2558.8 []+8922.0
Correlation coefficient	0.9999	0.9986
σ_{slope}	2.65	56.3
$\sigma_{intercept}$	1.36	2884
σ	349.4	7415
R.S.D. (%)	2.80 (11.68 ng/ml)	1.48 (11.68 ng/ml)
LOD (ng/ml)[a]	5.91	10.82

[a] Calculated by Clayton's method ($\alpha=\beta=0.05$).

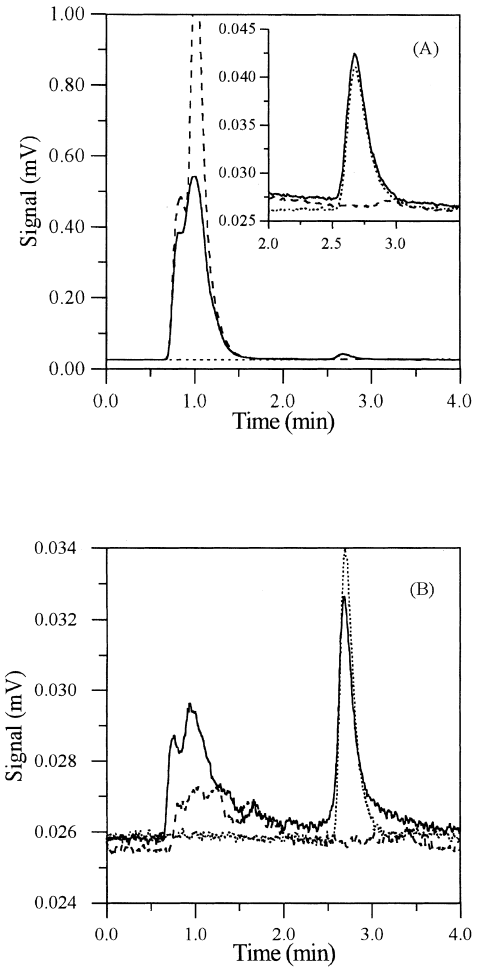

Fig. 4. (A) Chromatograms of: (———) Diluted urine sample spiked with 2.04 μg/ml of the antibiotics, (- - -) unspiked urine sample and (....) standard of piromidic acid 81.76 ng ml^{-1}. (B) Chromatograms of: (———) spiked trout muscle with the antibiotics (36 ng/g) treated according the proposed procedure, (- - -) unspiked trout muscle subjected to the proposed procedure and (....) standard of piromidic acid of 36 ng/ml.

presents the chromatogram obtained with a spiked urine sample together with the chromatogram of a unspiked urine sample. The results obtained are summarized in Table 2.

3.5.2. Trout muscle tissue

Different procedures have been proposed in the literature to analyze piromidic acid in the presence of other quinolonic antibiotics in fish muscle. Some involve the extraction of the drugs from the solid matrix with an appropriate solvent, liquid–liquid partition steps [5,6] or solid–liquid extraction [7,8,11,12] to obtain a cleaner sample. In these steps, the acid–base properties of these compounds are conveniently used and different aqueous phases are used [9,10]. In a previous paper [23], a simplified procedure was proposed that used the extraction of fish muscle with a strongly alkaline aqueous solution (sodium hydroxide) in which the drugs are soluble, followed by acidification and extraction with chloroform [3,5]. This is the procedure proposed in this instance to analyze the piromidic acid in trout muscle tissue. The extraction of piromidic acid into chloroform has been recently reported as complete (recoveries of 101%, in organic phase, for pH between 2.2 and 5.3) [24].

Table 2
Results obtained in the analysis of piromidic acid in spiked urine samples following the procedure described in Section 2, λ_{ex}=275 nm, λ_{em}=456 nm

[PIRO] added (μg/ml)	Recovery (%)[a]	
	Height	Area
0.99	95.7±0.8	87.7±0.9
1.52	103.6±1.0	98.0±1.1
2.04	102.8±0.6	98.4±0.8

[a] Mean recovery of three separate determinations.

Table 3
Recoveries obtained in the analysis of piromidic acid in spiked trout muscle tissue following the procedure described in Section 2

[PIRO] added (ng/g)	Recovery (%)[a]	
	Height	Area
33.5	72.3±1.5	76.8±1.7
65.0	77.0±0.9	80.5±1.2
100.0	78.9±1.3	79.8±1.4

[a] Mean recovery of three separate determinations.

The procedure has been used to analyze samples of trout muscle tissue spiked with piromidic acid (small quantities in chloroform). The solvent is carefully removed by a N_2 stream before the addition of the sodium hydroxide solution. The proposed method as described in Section 2, was then applied. Fig. 4B presents, as an example, the chromatograms obtained with a spiked trout muscle sample and an unspiked sample. Results obtained are shown in Table 3.

4. Conclusions

We propose, for the first time, the determination of piromidic acid by HPLC with fluorimetric detection. This is possible by means of a change of the pH of the eluted compounds, obtained with an additional NaOH stream post-column. This allows the analysis of piromidic acid in a short time without interference by other antibiotics of the same family. The sensitivity is substantially improved with respect to photometric detection [23]. The method is used to determine this quinolonic antibiotic in human urine without previous treatment and in trout muscle tissue. The recoveries obtained in all cases are very satisfactory.

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