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Application of terbium sensitized fluorescence for the determination of fluoroquinolone antibiotics pefloxacin, ciprofloxacin and norfloxacin in serum

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Abstract

A simple, rapid and sensitive spectrofluorimetric method for the determination of fluoroquinolone antibiotics, norfloxacin (NOR), ciprofloxacin (CIP) and pefloxacin (PEF) is described. The method is based on the radiative energy transfer from fluoroquinolones to terbium ions (Tb^{3+}) in the presence of tri-*n*-octylphosphine oxide (TOPO) in weakly acidic (pH 5.5) micellar solution of cetylpyridinium chloride (CPCl). Optimum conditions for the formation of the fluoroquinolone–Tb^{3+}–TOPO ternary complexes have been investigated. Under optimized conditions the detection limits are 1.7, 1.2 and 4.4 nM for NOR, CIP and PEF, respectively, while the range of application for all three drugs is 0.05–10 μM. The method has been successfully applied to the determination of NOR, CIP and PEF in serum samples after deproteinization with acetonitrile (serum–acetonitrile; 1:2, v/v). The mean recoveries from serum samples spiked with NOR, CIP and PEF (5.0–50.0 μM) were (90.3 ± 4.9), (105.0 ± 3.6) and (95.3 ± 1.5)%, respectively. Within-run and day-to-day s_r values for 5.0, 25.0 and 50.0 μM of each fluoroquinolone varied from 1.7 to 5.4% and from 3.3 to 12.8%, respectively. The influence of several usually coadministered drugs on the determination of fluoroquinolones in serum has been investigated. © 1997 Elsevier Science B.V.

Keywords: Terbium sensitized fluorescence; Fluoroquinolones; Norfloxacin; Ciprofloxacin; Pefloxacin; Serum

1. Introduction

The second generation of quinolone antibiotics, fluoroquinolones, are characterized by a wider antimicrobial spectrum and significant changes in their antibacterial potency, pharmacokinetic profiles and metabolic properties in comparison to their progenitor, nalidixic acid [1–3]. These changes are due to a number of modifications to the quinolone molecule at the N-1 and the C-6, C-7 and C-8 positions [4] (Scheme 1). Since the introduction of the first fluoroquinolone, norfloxacin, into clinical practice in the 1980s, this new group of antimicrobial agents is widely used in the treatment of severe systematic infections. There is a renewed interest in the development of new methods for the determination of fluoroquinolones at residue levels in complex biological samples. Among the techniques used for

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the determination of fluoroquinolones in biological fluids high performance liquid chromatography (HPLC) with direct fluorescence detection is the most widely used [5,6]. The ability of fluoroquinolones to form fluorescent complexes with metal ions such as aluminum [7,8] and terbium [9] has also been exploited for their detection. The formation of terbium chelates with fluoroquinolones was the basis for the development of a post-column reagent for the determination of ciprofloxacin by HPLC [9].

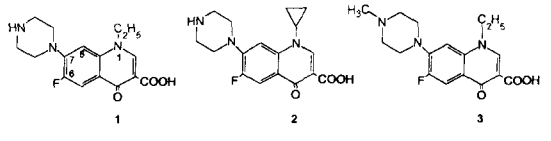
Terbium ions show unique fluorescent properties when complexed with organic ligands. The strong ion emission of these complexes originates from an intrachelate energy transfer from the triplet state of the ligand to the excited energy levels of the lanthanide ion. Methods for the selective and sensitive determination of several organic compounds, which serve as energy donors to lanthanides, have been developed [10].

Here we report a detailed study on the sensitization of terbium ion fluorescence by norfloxacin, ciprofloxacin and pefloxacin and the development of a simple, rapid and sensitive spectrofluorimetric method for their determination in serum samples.

2. Experimental

2.1. Apparatus

All fluorescence measurements were performed with a model 512-A double beam fluorescence spectrophotometer (Perkin-Elmer, Norwalk, CT) interfaced to an IBM-PC 386DX microcomputer for data acquisition and calculations [11]. Instrument settings were as follows: energy mode; excitation and emission monochromators bandwidths of 20 nm. A constant temperature of 25.0°C in the 1.000 cm (pathlength) sample cell was maintained with a thermostated water bath.



Scheme 1. Chemical structures of norfloxacin (1), ciprofloxacin (2) and pefloxacin (3).

2.2. Reagents and solutions

Fluoroquinolone antibiotics, norfloxacin, ciprofloxacin and pefloxacin were kindly donated by pharmaceutical companies. All other chemicals used were from Merck, (Darmstadt, Germany) and Sigma, (St. Louis, MO) unless otherwise stated.

Deionized, double-distilled water (DDW) was used to prepare all aqueous solutions. Aqueous stock solutions of quinolones (10 mM) were prepared and stored in the dark under refrigeration. Working standard solutions of quinolones were prepared daily by appropriate dilution with DDW or serum.

Aqueous stock solutions of Tb^{3+} (10 mM), CPCl (100 mM) and ethanolic solution of TOPO (10 mM) were also prepared. Acetate stock buffer solution (pH 5.5; 1.0 M) was employed. A working solution of Tb^{3+} (3.0 mM)–TOPO (1.5 mM)–CPCl (5 mM)–acetate (pH 5.5; 0.1 M) was prepared by appropriate mixing of the stock solutions and used as a single reagent.

2.3. Sample preparation

Serum samples and standards were prepared in control serum (normal) spiked with the appropriate quinolone in a concentration range of 5.0–50 μM. These samples were further deproteinized with acetonitrile, by mixing 100 μl of serum sample with 200 μl of acetonitrile, vortexing and centrifuging for 5 min at 1500 × *g*.

2.4. Procedure

100 μl of the deproteinized serum samples or standards were placed into the cuvette together with 1400 μl of working solution and the fluorescence intensity was measured versus a blank (λ_{ex} = 333 nm and λ_{em} = 546 nm) in which 100 μl of drug-free serum is substituted for standard or sample. The unknown concentration was calculated from the calibration graph.

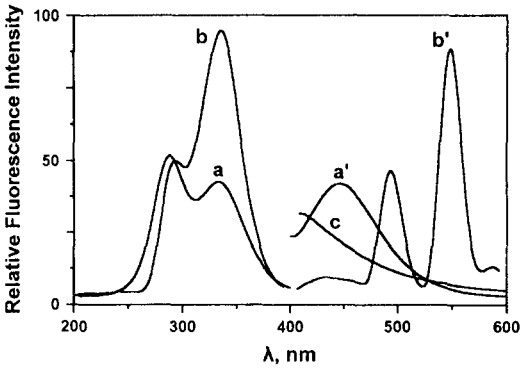


Fig. 1. Fluorescence excitation (a, b) and emission (a', b', c) spectra of CIP, CIP–Tb^{3+}–TOPO ternary complex and 100-fold diluted normal control serum, $C_{CIP} = 1$ μM, $C_{Tb} = 3$ mM, $C_{TOPO} = 1.5$ mM, $\lambda_{ex} = 333$ nm, $\lambda_{em} = 446$ nm (a, a'), $\lambda_{ex} = 333$ nm, $\lambda_{em} = 546$ nm (b, b') and $\lambda_{ex} = 333$ nm (c).

Non-treated serum samples were also measured by the following procedure: 15 μl of serum sample or standard were placed into the cuvette together with 1485 μl of working solution (100-fold final dilution of serum) and the fluorescence intensity was measured versus a blank, in which 15 μl of drug-free serum is substituted for the standard or sample.

3. Results and discussion

3.1. Luminescence spectra

Weakly acidic (pH 5.5) aqueous solutions of NOR, CIP and PEF show an intrinsic fluorescence ($\lambda_{ex} \approx 280$ nm, $\lambda_{em} \approx 440$ nm), which effectively decreases in the presence of Tb^{3+} and TOPO. At the same time a new emission band characteristic for terbium ion fluorescence in the range 450–610 nm (λ_{em}: 490, 546 and 590 nm) appears due to the energy transfer from the quinolone ligand to the emitting energy level of Tb^{3+}. Fig. 1 shows the characteristic excitation and emission spectra of the intrinsic fluorescence of CIP (curves a and a') and its ternary complex with Tb^{3+} and TOPO (curves b and b') together with the emission spectrum of a 100-fold diluted normal control serum (c). As can be seen from this spectra the fluorescence signal of the ternary complex of CIP is little influenced by the background fluorescence of serum, while the emission spectrum of its intrinsic fluorescence is in the range of the self-emission of the biological matrix.

3.2. Optimum conditions for quinolone–Tb^{3+}–TOPO complex formation

3.2.1. Effect of pH

Fluorescence intensity of quinolone–Tb^{3+}–TOPO complexes is pH dependent. Fig. 2 shows the variation of the fluorescence intensity with pH. The optimum pH for the formation of ternary complexes for all three quinolones, as expected, is about the same and ranges from 5.0 to 6.5. This is due to the quite similar $pK_{a,1}$ values of the carboxylate groups of CIP, NOR and PEF which together with the keto group are responsible for the complexation [7,9]. An acetate buffer solution of pH 5.5 at a concentration of 0.1 M was found to be suitable for the measurements.

3.2.2. Effect of terbium, TOPO and CPCl concentration

The effect of terbium concentration on the analytical signal for PEF–Tb^{3+}–TOPO ternary complex was studied. As can be seen from Fig. 3 the maximum fluorescence is observed at a terbium concentration above 2 mM. A terbium concentration of 3 mM was selected for the measurements. The presence of TOPO is essential, playing a protective role by creating an insulated sheath

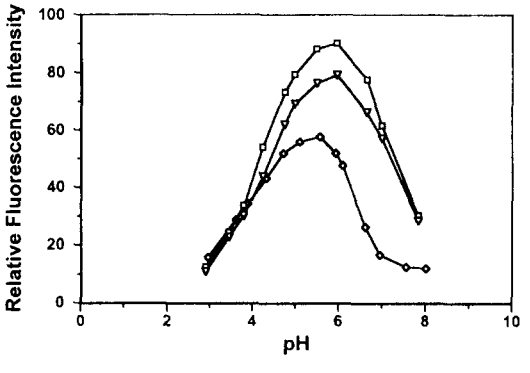


Fig. 2. Effect of pH on the fluorescence signal of the fluoroquinolone–Tb^{3+}–TOPO ternary complexes: NOR (▽), CIP (□) and PEF (◇). Other conditions as in Section 2.4.

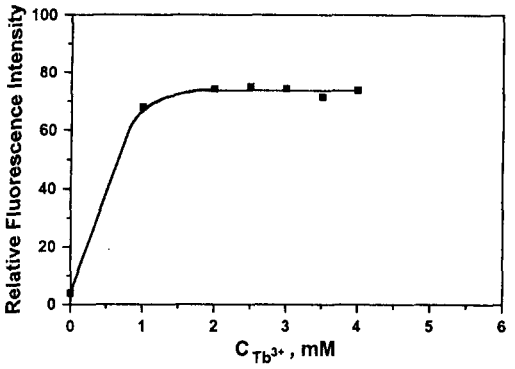


Fig. 3. Effect of terbium concentration on the fluorescence intensity of PEF–Tb^{3+}–TOPO ternary complex. Other conditions as in Section 2.4.

around Tb^{3+}, assuring the dehydration of the lanthanide ions [12]. Fig. 4 shows the effect of TOPO concentration on the fluorescence intensity of PEF–Tb^{3+}–TOPO complex. This study has been performed in the presence of cetylpyridinium chloride (CPCl) because of the poor solubility of TOPO in water. The maximum fluorescence was observed at 1.5 mM of TOPO and 5 mM of CPCl.

3.2.3. Analytical features

Under optimized conditions quinolone final concentration and relative fluorescence intensity were linearly related over the range 0.05–10 μM for all three quinolones. Pearson's correlation coefficients (r) for the calibration graphs were

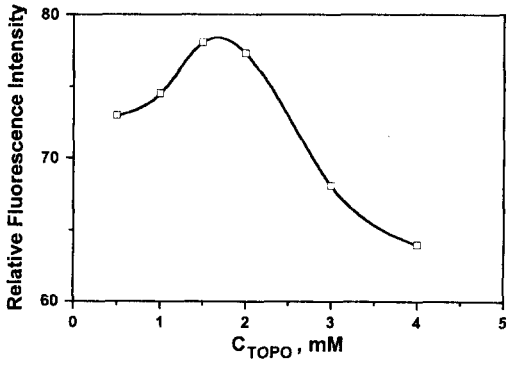


Fig. 4. Effect of TOPO concentration on the fluorescence intensity of PEF–Tb^{3+}–TOPO ternary complex. Other conditions as in Section 2.4.

Table 1
Analytical recoveries of NOR, CIP and PEF in serum samples[a]

Analyte	Added (μM)	Found[b] (μM)	Recovery (%)
NOR	5.0	4.8	96
	15.0	13.2	88
	30.0	26.0	87
CIP	5.0	5.3	106
	15.0	16.5	101
	30.0	32.5	108
PEF	5.0	4.7	94
	15.0	14.3	95
	30.0	29.1	97

[a] After deproteinization; [b] average of five measurements.

0.9992, 0.9996 and 0.999 for NOR, CIP and PEF respectively. The detection limits (DOLs), defined as the concentration corresponding to a signal equal to three times the S.D. of the blank, were 1.7, 1.2 and 4.4 nM for NOR, CIP and PEF, respectively.

3.2.4. Serum samples

A detailed study on the influence of serum matrix on the proposed method has been performed. For this purpose, serum samples and standards were measured either without any pretreatment or after deproteinization with acetonitrile. The slope of the calibration graphs for all three drugs obtained by using either non-treated serum (1:100 final dilution) or aqueous standards was practically the same. The background fluorescence of the serum, however, was relatively high (signal-to-noise ratio of about 1.3). Taking into account the limitations towards lower dilutions of the serum (reaction mixture became turbid) this fact limits the application range of the method towards lower concentrations of the drug in serum. The slope of the calibration graphs for all three drugs obtained by using deproteinized serum samples was found to be about 30% lower than that of aqueous standards. This could be explained by the partial deactivation of the terbium complexes in the reaction mixture due to the presence of organic solvent (acetonitrile) with a dielectric constant lower than that of water [13]. However, in this case the background fluorescence

Table 2
Precision data

Analyte	Mean concentration[a] (μM)	Within-run precision (s_r %)	Mean concentration[b] (μM)	Day-to-day precision (s_r %)
NOR	4.8	5.4	4.6	10.5
	26.4	3.6	14.0	3.3
	51.5	4.8	27.6	6.7
CIP	4.7	3.4	4.9	8.4
	25.5	3.4	14.5	6.2
	49.8	1.7	29.5	5.2
PEF	4.8	4.2	4.7	12.8
	23.7	3.5	14.6	8.0
	51.9	2.6	30.5	7.5

[a] Average of five measurements; [b] average of five measurements.

of deproteinized serum was about half of that of the non-treated sample despite the lower final dilution of the sample (1:45) and was practically the same even when 300 μl of the serum supernatant was used for the measurements (final dilution of the sample 1:15) and thus improving the detection limit of the method one order of magnitude. The method has been successfully applied to the determination of NOR, CIP and PEF in serum samples after simple deproteinization with acetonitrile. By using 100 μl of deproteinized serum the DOLs were found to be 0.2, 0.1 and 0.3 μM for NOR, CIP and PEF, respectively. It should be emphasized that the DOLs easily meet the sensitivity requirements for the determination of fluoroquinolones in serum.

3.2.5. Recovery and precision

Analytical recovery was assessed by analyzing serum samples spiked with fluoroquinolone at three different concentrations and the results are shown in Table 1. To determine within-run precision, three serum pools containing different concentrations of each fluoroquinolone were measured five times each. To assess day-to-day precision repeated analyses of three serum samples over two weeks were performed and the results are shown in Table 2. As can be seen from Table 1, analytical recoveries were quite satisfactory for all three drugs while day-to-day precision data (Table 2) show that serum samples can be stored at 4°C for at least 2 weeks before final measurement.

3.2.6. Effect of foreign substances

The influence of twenty usually coadministered drugs on the determination of fluoroquinolones has been investigated. Amounts of drugs under investigation were added to serum samples spiked with CIP (5.0 μM) at a final concentration of 0.3 g l^{-1}. Phenobarbital, cefadroxil, phenytoin, gentamicin, lorazepam, diazepam, prazepam, trimethoprim, sulfamethoxazole, sulfisoxazole, acetaminophen, amikacin, warfarine, thiamine, digitoxin, cimetidine and cefatrizine do not interfere with the determination of CIP. Salicylates and furosemide caused positive errors (more than ± 5% change in the fluorescence intensity) at mass ratios (drug to CIP) > 1:30, while ranitidine caused negative errors at mass ratios > 1:40.

4. Conclusions

The proposed spectrofluorimetric method for the determination of NOR, CIP and PEF in serum is very simple, rapid and sensitive and could be easily automated. The proposed chemical system could also been exploited as a post-column detection system for the HPLC determination of the above mentioned drugs.

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