Determination of the antibacterial norfloxacin in human urine and serum samples by solid-phase spectrofluorimetry

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Abstract

A method for the determination of trace amounts of norfloxacin has been developed, based on solid-phase spectrofluorimetry. The relative fluorescence intensity (RFI) of norfloxacin fixed on Sephadex SP C-25 gel was measured directly after packing the gel beads in a 1 mm silica cell, using a solid-phase attachment. The wavelengths of excitation and emission were 272 and 446 nm, respectively. Using a sample volume of 1000 ml, the linear concentration range of application was 0.1–4.0 ng ml$^{-1}$ of norfloxacin, with a relative standard deviation (R.S.D.) of 1.4% (for a level of 2.0 ng ml$^{-1}$) and a detection limit of 0.04 ng ml$^{-1}$.

The method was applied to the determination of norfloxacin in human urine and serum samples. It was validated applying the standard addition methodology and using HPLC as a reference method. Recovery levels of the method reached 100% in all cases. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Norfloxacin; Solid-phase spectrofluorimetry; Urine analysis; Serum analysis

1. Introduction

Fluoroquinolones are a class of important antibacterials, which have been developed rapidly in recent years and have wide applications in clinical medicine. Norfloxacin (NFLX) [1-ethyl-6-fluoro-1,4-dihydro-4-oxo-7-(1-piperazinyl)-3-quinoline-carboxylic acid] (Fig. 1) is a synthetic fluoroquinolone derivative, which has demonstrated broad-spectrum activity against many pathogenic gram-negative and gram-positive bacteria. The bactericidal action of NFLX results from interference with enzyme DNA gyrase which is needed for the synthesis of bacterial DNA [1,2].

This synthetic antibiotic is widely used in the treatment of urinary infections with good localised action on infected sites. About 35% of the oral dose is excreted unchanged in the urine within 24 h, thus producing high urinary concentrations. Different techniques have been proposed for the determination of the drug. NFLX has been determined by polargraphy, adsorptive stripping voltammetry and high-performance liquid chromatography (HPLC) [3–10].

Extraction spectrophotometric procedures using Bromothymol Blue, tetrachloro-benzoquinone and Supracen Violet 3B have also been described [11–15]. NFLX forms chelates with Th(III), Fe(III) and Al(III), enabling thus the photometric and fluorimetric determination of the drug [16–21].

Solid-phase spectrofluorimetry (SPF) has been found to be useful for the determination of trace...
amounts of different inorganic compounds [22–25] and organic compounds [26–33] in real samples, showing several advantages such as: low interference level, low detection limit, high sensitivity and the use of conventional instrumentation.

A method for determination of trace amounts of NFLX by SPF is described in this paper, having been satisfactorily applied to the determination of NFLX in human urine and serum samples.

2. Experimental

2.1. Apparatus and software

A Perkin-Elmer LS-50 luminescence spectrometer fitted with accessories described previously [24] was used to perform all spectrofluorimetric measurements, and a variable-angle surface accessory designed and constructed by the authors [24] was also used to carry out the measurements of relative fluorescence intensity (RFI) in gel phase.

A Cinson 501 digital pH-meter with a combined glass-saturated calomel electrode and an Agitaser 2000 rotating agitator were also used. The Statgraphics [34] software package was used for the statistical analysis of data.

2.2. Reagents

All reagents were of the analytical-reagent grade unless stated otherwise. Water was purified with a Milli-Q plus system (Millipore).

Sephadex SP C-25 dextran type cation-exchange gel (Sigma) was used in the original dry state obtained from the supplier and without any pre-treatment in order to avoid contamination.

Norfloxacin stock standard solution (0.1 mg ml⁻¹) was prepared by exact weighing of reagent (Sigma) and dissolution in deionised water. This solution was stable for at least 1 week, if stored in the dark at 4°C. Working solutions were prepared by appropriate dilutions with deionised water.

Buffer solutions of required pH were made from 0.5 M sodium acetate (Merck) solution and 0.5 M acetic acid (Merck) solution.

2.3. Fluorescence measurements

The measured RFI of the gel beads containing the fluorescent analyte and packed into a 1 mm silica cell was the diffuse transmitted fluorescence emitted from the gel at the unirradiated face of the cell. The optimum angle between the cell plane and the excitation beam was 45° in all instances [24].

2.4. Basic procedure

To an aliquot of the sample containing between 0.1 and 4.0 μg of NFLX, 20 ml of 0.5 M acetate buffer solution (pH 3.8) were added and made up to 1000 ml with deionised water. The solution was transferred into a 2 l glass bottle and 50 mg of Sephadex SP C-25 gel were added. The mixture was shaken mechanically for 5 min, after which the gel beads were collected by filtration under suction and, with the aid of a pipette, were packed into a 1 mm silica cell together with a small volume of the filtrate (0.2 ml). A blank sample containing all reagents except NFLX was prepared and treated in the same way as described for the sample.

The fluorescence intensities (20.0 ± 0.5°C) of the sample and blank were always measured at λex = 446 nm with λem = 272 nm. The calibration graph was constructed in the same way with NFLX solutions of known concentrations.

2.5. Sample treatment

Urine samples were obtained from fasting healthy men and the serum samples were supplied by the “Virgen de las Nieves” Hospital (Granada). The samples were centrifuged for 10 min at 3800 rpm and filtered through a cellulose acetate filter (0.45 μm pore size, Millipore HA WP 04700). The filtrates were collected in glass containers that had been carefully cleaned with
hydrochloric acid and washed with deionised water and stored at 4°C until analysis was performed, with the minimum possible delay. Aliquots of these filtrates (0.1 ml for urine samples and 1.0 ml for serum sample, respectively) were taken and treated as described in Section 2.4.

3. Results and discussion

3.1. Spectral characteristics

NFLX shows native fluorescence in aqueous solutions. The influence of pH on its fluorescence intensity has been studied by different authors [35,36]. From these works, it was observed that the maximum fluorescence emission is obtained in the pH interval 4–7. This is behaviour attributed by the authors to the appearance of a zwitterionic form due to the progressive ionisation of the carboxylic group. Norfloxacin present two values of $pK_a$ (6.22 and 8.38) corresponding at the carboxylic group and at the piperazinyl group, respectively.

In the presence of Sephadex cation-exchange gel, NFLX is fixed on the gel, as NFLX is not fixed on anion-exchange gels. A SP C-25 dextran-type gel was selected as it was found to have a less background fluorescence.

Norfloxacin in the presence of Sephadex SP C-25 gel is fixed at a slightly acid pH (Fig. 2). The peak wavelengths in the excitation spectra of NFLX are 278 and 325 nm in solution; 272 and 320 nm in the gel phase. Also, the maximum of the emission spectra for the two systems differ, being located at 445 nm in solution and 448 nm in the gel phase. The modification of the features of the fluorescence spectra was considered to be a result of the modification of the surrounding environment of the compound in the gel phase with respect to solution.

From a study of the half-life of the excited state of the compound in the solid phase at different temperatures, it was concluded that the luminescence process was fluorescence ($\tau < 5 \times 10^{-6}$ s).

3.2. Effect of experimental variables

3.2.1. pH dependence

The RFI of NFLX fixed on the gel is a maximum in the pH interval 3.5–4.2 (Fig. 3). Different buffer solutions (acetate, monochloroacetate, phosphate and Britton-Robinson) were tested. Sodium acetate/acetic acid buffer solution (pH 3.8) was found to be the most successful. Changes in the buffer concentration produced little variation in the observed fluorescence intensity. A 0.01 M concentration of the buffer was selected to obtain an
Fig. 3. Influence of pH on RFI of norfloxacin fixed on Sephadex SP C-25: λ
exc = 272 nm; λ
em = 446 nm; slit exc = 5 nm; slit em = 5 nm; v = 480 nm min
−1; sample volume = 500 ml; [NFLX] = 3.0 ng ml
−1. Adequate buffering capacity without excessive loss of sensitivity.

The fluorescence is independent of ionic strength, adjusted with the buffer solution, NaCl or NaClO4, up to 0.01 M, decreasing for higher values.

3.2.2. Influence of temperature
The RFI dependence on temperature of NFLX fixed on the gel is small (−0.6 %°C
−1 between 5 and 70 °C). The RFI decrease with temperature was totally reversible. All RFI measurements reported here were performed at 20.0 ± 0.5 °C. Since the fixation process was independent of temperature in the range 0–40 °C, the fixation of NFLX on the gel was carried out at room temperature.

3.2.3. Effect of the sample volume on the sensitivity
Previous reports [22–33] indicated that the main advantages of SPF methods are the potential increase in sensitivity with increase in sample volume taken for analysis. This effect can be assessed by measuring of the RFI of Sephadex SP C-25 gel equilibrated with different volumes of solutions containing the same concentration of NFLX and proportional amounts of other reagents.

Plots of RFI versus sample volume show an increase in fluorescence signal with sample volume, tending asymptotically to a constant RFI value above a certain volume (1000 ml in this study). The shape of the graphs suggested a Langmuir-type isotherm, as was observed in some other SPF studies [24,29–33].

In practice, this increase in sensitivity can be calculated from the slope of the calibration graphs. The calculated values of the sensitivity ratio (S) for the samples analysed in this study were: S1500/250 = 2.7; S1000/250 = 2.6; and S500/250 = 1.3, where the subscripts represent the sample volume (ml). The non-linear dependence of sensitivity versus sample volume can be attributed to the decrease in the distribution coefficient with analyte concentration, as is usual in a non-linear isotherm.

3.2.4. Other experimental conditions
The stirring time necessary for maximum RFI development was 5 min. Longer times did not result in any improvement. As the use of a large amount of gel lowered the RFI, only the amount required to fill the cell and facilitate handling, i.e. 50 mg, was used in all measurements.

With regard to the stability of the SP C-25 gel-NFLX system, the RFI remained constant for at least 1 h. Order of addition of the reagents (sample and buffer solution) did not affect the results obtained.

3.3. Analytical parameters
The calibration graphs for the samples treated according to the procedure described above are linear (0.1–4.0 ng ml
−1 as concentration range. The lack-of-fit test [37] was used to check the linearity of the calibration graphs. Six replicates were used for each of five standards prepared to obtain the calibration graph.

The IUPAC detection limit [38] found was 0.04 ng ml
−1 and the quantification limit was 0.14 ng ml
−1.

The repeatability of the present method and of the packing of the gel in the 1 mm silica cell was also determined. The precision was measured for NFLX concentrations of 1.0, 2.0 and 3.0 ng ml
−1, by performing 10 independent determinations. Relative standard deviations (R.S.D.) were 1.6, 1.4 and 1.1%, respectively. The precision (R.S.D.) of the packing operation, calculated from 10 measurements, was 1.0% for the NFLX fixed on the gel and 0.9% for the gel blank. The precision (R.S.D.) of the fluorescence measurements (noise) was about 0.5%. The analytical parameters are summarised in Table 1.
The increase in sensitivity obtained with the proposed method, respect to other methods is very substantial. Comparison with other proposed methods for the determination of norfloxacin, described in the literature (Table 2), shows an improvement of about one-order of magnitude versus HPLC methods. The proposed method is the most sensitive reported to date. Furthermore, the HPLC methods require more analysis total time since these methods need an extraction step previous of the analytes in the samples. The proposed method pre-concentrate and extract in the same step.

3.4. Effect of foreign species

To evaluate the potential effect of foreign ionic species, commonly found in urine and serum, on the determination of NFLX at 2 ng ml\(^{-1}\) level, a systematic study was carried out. A 20 \(\mu\)g ml\(^{-1}\) level of potentially interfering species was tested first, and if interference occurred the ratio was reduced progressively until interference ceased. Tolerance was defined as the amount of foreign species that produced an error not exceeding \(\pm 5\%\) in the determination of the analyte. Table 3 shows the results obtained.

3.5. Validation and application of the method

3.5.1. Spiked samples

The validation of the proposed method was carried out on spiked samples of human urine and serum (final norfloxacin concentration of 10.0 and 0.5 \(\mu\)g ml\(^{-1}\) for urine and serum, respectively) using the standard addition methodology [39]. Whereby three experiments are required to obtain the data set necessary to carry out the statistical protocol: (a) standard calibration (SC) as described above; (b) standard addition calibration (AC), which is obtained by addition of continuous variations of standard at constant sample volume; (c) Youden calibration (YC), with the Youden method [40] in which a calibration curve is established with continuous variation of sample volume.

By applying linear regression analysis, the slope, the intercept and the regression standard deviation for...
Table 4
Values numerical of parameters of SC, AC and YC for urine

<table>
<thead>
<tr>
<th>Parameter</th>
<th>SC</th>
<th>AC</th>
<th>YC</th>
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</thead>
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<tr>
<td>Calibration</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>(n)</td>
<td>30</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>(a)</td>
<td>1.22</td>
<td>100.44</td>
<td>–0.13</td>
</tr>
<tr>
<td>(b)</td>
<td>99.71</td>
<td>99.82</td>
<td>0.996</td>
</tr>
<tr>
<td>(s)</td>
<td>2.68</td>
<td>3.11</td>
<td>3.52</td>
</tr>
<tr>
<td>(s_p)</td>
<td>2.75</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(t(b))</td>
<td>99.71</td>
<td>99.82</td>
<td>0.996</td>
</tr>
<tr>
<td>(YB)</td>
<td>–1.34</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Analysis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(C_x (\text{ng ml}^{-1}))</td>
<td>1.03</td>
<td>1.01</td>
<td></td>
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<tr>
<td>(c_\text{umk} (\mu\text{g ml}^{-1}))</td>
<td>10.26</td>
<td>10.10</td>
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<tr>
<td>(9.99)</td>
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<td></td>
<td></td>
</tr>
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</table>

*Results of analytic contents to check accuracy.

\(n\): numbers of measurements; \(a\): intercept; \(b\): slope; \(s\): regression standard deviation; \(s_p\): pooled regression standard deviation; \(t(b)\): \(t\)-value for \(nSC + nAC - 4\) degrees of freedom at \(P = 1\%\) level; \(YB\): Youden blank.

Critical value 2.72 (1%); \(P\): percentage of Student’s \(t\)-distribution.

Table 5
Values numerical of parameters of SC, AC and YC for serum

<table>
<thead>
<tr>
<th>Parameter</th>
<th>SC</th>
<th>AC</th>
<th>YC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calibration</td>
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</tr>
<tr>
<td>(n)</td>
<td>30</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>(a)</td>
<td>1.72</td>
<td>49.30</td>
<td>1.50</td>
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<tr>
<td>(b)</td>
<td>99.71</td>
<td>99.63</td>
<td>0.049</td>
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<tr>
<td>(s)</td>
<td>2.68</td>
<td>2.35</td>
<td>1.87</td>
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<tr>
<td>(s_p)</td>
<td>2.63</td>
<td></td>
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<tr>
<td>(t(b))</td>
<td>99.70</td>
<td>99.70</td>
<td></td>
</tr>
<tr>
<td>(YB)</td>
<td>–1.34</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Analysis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(C_x (\text{ng ml}^{-1}))</td>
<td>0.484</td>
<td>0.479</td>
<td></td>
</tr>
<tr>
<td>(c_\text{umk} (\mu\text{g ml}^{-1}))</td>
<td>0.48</td>
<td>0.48</td>
<td></td>
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<tr>
<td>(9.99)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Results of analytic contents to check accuracy.

\(n\): numbers of measurements; \(a\): intercept; \(b\): slope; \(s\): regression standard deviation; \(s_p\): pooled regression standard deviation; \(t(b)\): \(t\)-value for \(nSC + nAC - 4\) degrees of freedom at \(P = 1\%\) level; \(YB\): Youden blank.

Critical value 2.72 (1%); \(P\): percentage of Student’s \(t\)-distribution.

Table 6
Results of recovery assays to check the accuracy of the proposed method

<table>
<thead>
<tr>
<th>Sample</th>
<th>Spiked* (\mu\text{g ml}^{-1})</th>
<th>Found* (\mu\text{g ml}^{-1})</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human urine-1</td>
<td>5</td>
<td>5.1 ± 0.2</td>
<td>101.0</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>9.7 ± 0.2</td>
<td>97.0</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>15.2 ± 0.3</td>
<td>101.3</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>20.3 ± 0.3</td>
<td>101.5</td>
</tr>
<tr>
<td>Human urine-2</td>
<td>5</td>
<td>4.8 ± 0.2</td>
<td>96.0</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>10.2 ± 0.2</td>
<td>102.0</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>14.9 ± 0.2</td>
<td>99.3</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>19.8 ± 0.3</td>
<td>99.0</td>
</tr>
<tr>
<td>Human serum</td>
<td>0.4</td>
<td>0.39 ± 0.02</td>
<td>97.5</td>
</tr>
<tr>
<td></td>
<td>0.8</td>
<td>0.77 ± 0.02</td>
<td>96.3</td>
</tr>
<tr>
<td></td>
<td>1.2</td>
<td>1.22 ± 0.03</td>
<td>101.7</td>
</tr>
<tr>
<td></td>
<td>1.6</td>
<td>1.59 ± 0.03</td>
<td>100.4</td>
</tr>
</tbody>
</table>

*Using the Student’s \(t\)-test: \(R = 100.2\%), \(x_g = 3.58\), \(t(R) = 0.052\) (\(P = 48.1\%\)), critical value 3.182 (5%) for human urine-1; \(R = 99.1\%), \(x_g = 3.82\), \(t(R) = 0.202\) (\(P = 42.6\%\)), critical value 3.182 (5%) for human urine-2; and \(R = 98.7\%), \(x_g = 3.35\), \(t(R) = 0.226\) (\(P = 41.8\%\)), critical value 3.182 (5%) for human serum.

*Referred to original sample.

*Average value ± standard deviation of six determinations.
Table 7
Determination of norfloxacin in human urine samples

<table>
<thead>
<tr>
<th>Sample</th>
<th>Norfloxacin found (µg ml⁻¹) Proposed method</th>
<th>HPLC method</th>
<th>t</th>
<th>P-value (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human urine-1</td>
<td>48.1 ± 0.3</td>
<td>48.0 ± 0.2</td>
<td>1.40</td>
<td>18.0</td>
</tr>
<tr>
<td>Human urine-2</td>
<td>33.6 ± 0.2</td>
<td>33.7 ± 0.2</td>
<td>1.07</td>
<td>30.1</td>
</tr>
<tr>
<td>Human urine-3</td>
<td>38.8 ± 0.3</td>
<td>38.7 ± 0.2</td>
<td>1.68</td>
<td>11.1</td>
</tr>
</tbody>
</table>

a Urinary volume: 1.5 l for human urine-1 sample; 2.0 l for human urine-2 sample and 1.7 l for human urine-3 sample.

b Average value ± S.D. of nine determinations.

c P-value of the comparison test.

### 4. Conclusions

A sensitive, quickly and easy solid-phase spectrofluorimetric method is presented for the determination of norfloxacin in human urine and serum samples (0.1–4.0 µg l⁻¹), without the need of a pre-separation step. The detection limit obtained, 0.04 µg l⁻¹, was the lowest reported up to date. It was applied satisfactorily to analysis of biological samples with good recovery rates in all cases. The method was validated by comparison with a HPLC method.

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