ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
30 (2002) 1103–1110

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

www.elsevier.com/locate/jpba

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

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Received 19 February 2002; received in revised form 24 June 2002; accepted 7 July 2002

Abstract

A method for the determination of trace amounts of ofloxacin has been developed, based on solid-phase spectrofluorimetry. The relative fluorescence intensity of ofloxacin 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 294 and 494 nm, respectively. The linear concentration range of application was 0.5–16.0 ng ml^{-1} of ofloxacin, with a relative standard deviation of 1.1% (for a level of 8.0 ng ml^{-1}) and a detection limit of 0.14 ng ml^{-1}. The method was applied to the determination of ofloxacin 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.

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

1. Introduction

Quinolones have emerged as one of the most important classes of antibiotics of the past decade. Ofloxacin (OFLX) {9-fluoro-2,3-dihydro-3-methyl-10-(-methyl-1-piperzinyl)-7-oxo-7H-pyrido-[1,2,3-de]1,4-bonzoxazine-6-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 OFLX results from interference with enzyme DNA gyrase that 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 75% 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. OFLX has been determined by potenciometry, conductometry, differential-pulse voltammetry, adsorptive stripping voltammetry and high-performance liquid chromatography (HPLC) [3–11].

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0731-7085/02/$ - see front matter
PII: S0731-7085(02)00466-1

This drug has been also determined by spectrophotometric, spectrofluorimetric and flow-injection chemiluminesce [12–15]. Extraction spectrophotometric procedures using Bromophenol Blue, Bromothymol Blue, Bromocresol Purple and Tropaeolin 000 (TP 000) have also been described [16,17].

OFLX forms chelates with Fe(III) and Sc(III), enabling thus the photometric and fluorimetric determination of the drug [18–20].

Solid-phase spectrofluorimetry (SPF) has been found to be useful for the determination of trace amounts of different inorganic compounds [21–24] and organic compounds [25–32] 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 OFLX by SPF is described in this paper, having been satisfactorily applied in the determination of OFLX in human urine and serum samples.

The proposed method is the most sensitive reported to date and requires minor analysis total time than HPLC methods because this method pre-concentrates and extracts in the same step.

2. Experimental

2.1. Apparatus and software

A Perkin-Elmer (Norwalk, CT, USA) LS-50 luminescence spectrometer fitted with accessories described previously [23] was used to perform all spectrofluorimetric measurements, and a variable-angle surface accessory designed and constructed by the authors [23] was also used to carry out the measurements of relative fluorescence intensity (RFI) in gel phase.

A Crison (Barcelona, Spain) 501 digital pH-meter with a combined glass-saturated calomel electrode and an Agitaser (Barcelona, Spain) 2000 rotating agitator, were also used.

The Statgraphics [33] 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, Bedford, MA, USA).

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

Ofloxacin 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, Darmstadt, Germany) 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 [23].

2.4. Basic procedure

To an aliquot of the sample containing between 0.5 and 16.0 μg of OFLX, 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 OFLX was prepared and treated in the same way as described for the sample.

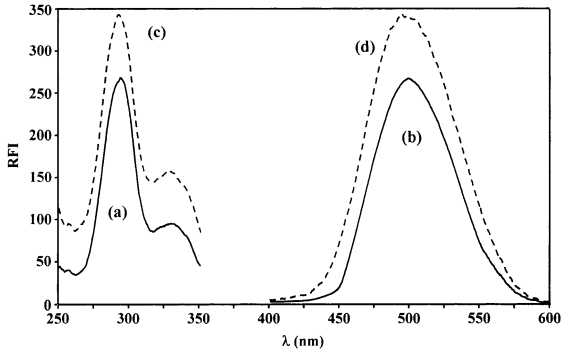

Fig. 1. Fluorescence spectra of: ofloxacin fixed on Sephadex SP C-25 (—), (a) excitation and (b) emission, [OFL] = 5.0 ng ml^{-1}, sample volume, 500 ml; ofloxacin in solution watery (· · ·), (c) excitation and (d) emission, [OFLX] = 1.0 mg l^{-1} (RFI, relative fluorescence intensity). $slit_{ex}$, 5 nm; $slit_{em}$, 5 nm; v, 480 nm min^{-1}.

The fluorescence intensities (20.0 ± 0.5 °C) of the sample and blank were always measured at $\lambda_{em} = 494$ nm with $\lambda_{ex} = 294$ nm. The calibration graph was constructed in the same way with OFLX 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 2.0 ml for serum samples, respectively) were taken and treated as described in Section 2.4.

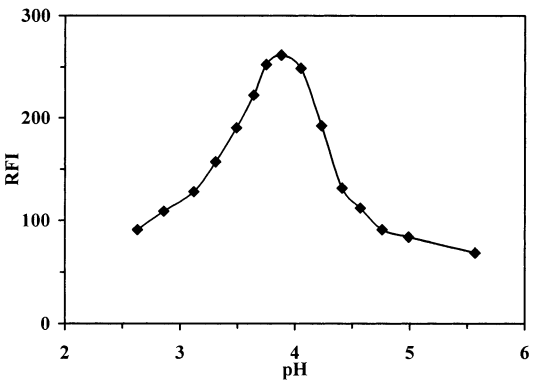

Fig. 2. Influence of pH on RFI of ofloxacin fixed on Sephadex SP C-25 (RFI, relative fluorescence intensity). λ_{ex}, 294 nm; λ_{em}, 494 nm; $slit_{ex}$, 5 nm; $slit_{em}$, 5 nm; sample volume, 500 ml; [OFLX] = 5.0 ng ml^{-1}.

3. Results and discussion

3.1. Spectral characteristics

OFLX shows native fluorescence in aqueous solutions. The influence of pH on its fluorescence intensity has been studied by different authors [18,20]. From these works, it was observed that the maximum fluorescence emission is obtained in the pH interval 2.5–4.5 and that this compound present two values of pK_a (6.05 and 8.22) corresponding at the carboxylic group and at the piperazinyl group, respectively.

In the presence of Sephadex cation-exchange gel, OFLX is fixed on the gel, as OFLX 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.

Ofloxacin 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 OFLX are identical for the immobilised and solvated systems (294 and 327 nm). The maximum of the emission spectra for the two systems differ, being located at 498 nm in solution and 494 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 relative fluorescence intensity of OFLX fixed on the gel is a maximum in the pH interval 3.5–4.2 (Fig. 2). 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 adequate buffering capacity without excessive loss of sensitivity.

The fluorescence is independent of ionic strength, adjusted with the buffer solution, NaCl or $NaClO_4$, up to 0.01 M, decreasing for higher values.

3.2.2. Influence of temperature

The RFI dependence on temperature of OFLX fixed on the gel is small ($-0.3\%\ ^{\circ}C^{-1}$ 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 OFLX on the gel was carried out at room temperature.

3.2.3. Effect of the sample volume on the sensitivity

Previous reports [20–31] indicated that the main of SPF methods advantages is the potential increase in sensitivity with increase in sample volume taken for analysis. This effect can be assessed by measuring the RFI of Sephadex SP C-25 gel equilibrated with different volumes of solutions containing the same concentration of OFLX 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 [23,28–32].

Table 1
Analytical parameters

Parameter	
Intercept	0.80
Slope (ml ng^{-1})	39.78
Linear dynamic range (ng ml^{-1})	0.5–16.0
Correlation coefficient	0.9998
P_{lof} (%)[a]	66.4
Detection limit[b] (ng ml^{-1})	0.14
Quantification limit (ng ml^{-1})	0.48

[a] Probability level of *lack-of-fit* test.
[b] For $\alpha = 5\%$, $\beta = 5\%$ and six replicates.

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: $S_{1500/250} = 2.0$, $S_{1000/250} = 1.9$, $S_{500/250} = 1.2$, 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-OFLX 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 of 0.5–16.0 ng ml^{-1} as concentration range. The *lack-of-fit* test [34] 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.

Table 2
Methods for the determination of ofloxacin

Technique	Application	Detection limit (ng ml^{-1})	Reference
DDP	Commercial formulations	108	[5]
DSF	Urine	72	[14]
HPLC-UV	Serum and urine	30	[11]
	Serum and urine	20	[8]
HPLC-FD	Plasma	6	[10]
AdSV	Commercial formulations	1	[6]
SPF	Serum and urine	0.14	This work

DPP, differential-pulse polarography; DSF, first-derivative synchronous spectrofluorimetry; HPLC-UV, high-performance liquid chromatography-ultraviolet detection; HPLC-FD, high-performance liquid chromatography-fluorimetric detection; AdSV, adsorptive differential-pulse stripping voltammetry; SPF, solid-phase spectrofluorimetry.

The IUPAC detection limit [35] found was 0.14 ng ml^{-1} and the quantification limit was 0.48 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 OFLX concentrations of 1.0, 4.0 and 8.0 ng ml^{-1}, by performing ten independent determinations. The relative standard deviations (R.S.D.) were 1.5, 1.3 and 1.1%, respectively. The precision (R.S.D.) of the packing operation, calculated from ten measurements, was 0.9% for the OFLX fixed on the gel and 1.0% 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 method reported here is substantially more sensitive that the solution methods based in the native fluorescence of the ofloxacin. So, this method is 100 times more sensitive that the method in solution under our experimental conditions (comparison of the slopes of calibration curves) and the detection limit (0.14 ng ml^{-1}) is two-order of magnitude minor than the detection limit found in solution.

Also, the method is compared in Table 2 with methods described in literature for the determination of OFLX. For comparison purposes we

Table 3
Effect of foreign species and co-administered drugs on the determination of 2.0 ng ml^{-1} of ofloxacin

Foreign species or compound	Tolerance level (ng·ml^{-1})
Cl^-	> 20 000
Mg(II)	4000
Ca(II)	600
Cu(II)	400
Paracetamol, aspirin	40
Amikacin, sulbactam, penicullin	20
Imipenem	10
Fe(III)	3.0

Table 4
Values numerical of parameters of SC, AC and YC for urine[a]

Parameter[b]	SC	AC	YC
Calibration			
n	36	7	6
a	0.80	161.96	2.77
b	3.78	39.90	1.581
s	3.70	3.89	2.97
s_p		3.63	
$t(b)$		0.251[c] (p = 80.3%)	
b_p		39.79	
a'	0.74	162.54	
YB			2.10
Analysis			
C_x (ng ml^{-1})	4.06	4.01	
		$t(c)$ = 1.31 (p = 19.9%)	
		Critical value 2.02 (5%)	
C_{sample} (μg ml^{-1})	40.63	40.14	39.74

[a] Results of analyte contents to check accuracy.
[b] n, numbers of measurements; a, intercept; b, slope; s, regression standard deviation; s_p, pooled regression standard deviation; $t(b)$, t-value for $n_{SC}+n_{AC}-4$ degrees of freedom at p = 1% level; b_p, pooled slope of SC and AC; a', new intercept; YB, Youden blank.
[c] Critical value 2.71 (1%); p, percentage of Student t-distribution.

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

Parameter[b]	SC	AC	YC
Calibration			
n	36	7	6
a	0.80	40.57	−0.06
b	39.78	39.68	0.020
s	3.70	3.29	1.53
s_p		3.65	
$t(b)$		0.154[c] ($p = 87.8\%$)	
b_p	39.78		
a'	0.84	40.2	
YB			−0.89
Analysis			
C_x (ng ml^{-1})	1.02	1.01	
		$t(c) = 0.259$ ($p = 79.7\%$)	
		Critical value 2.02 (5%)	
C_{sample} (μg ml^{-1})	0.511	0.506	0.505

[a] Results of analyte contents to check accuracy.
[b] n, numbers of measurements; a, intercept; b, slope; s, regression standard deviation; s_p, pooled regression standard deviation; $t(b)$, t-value for $n_{SC}+n_{AC}-4$ degrees of freedom at $p = 1\%$ level; b_p, pooled slope of SC and AC; a', new intercept; YB, Youden blank.
[c] Critical value 2.71 (1%); p, percentage of Student t-distribution.

selected those methods which we considered to be the most sensitive reported to date. The reported data show an improvement of about one-order of magnitude versus HPLC methods.

3.4. Effect of foreign species

To evaluate the potential effect of foreign ionic species, commonly found in urine and serum, and co-administered drugs on the determination of OFLX at 2 ng ml^{-1} level, a systematic study was carried out. A 20 μg 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 ±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 ofloxacin concentration of 40.0 and 0.5 μg ml^{-1} for urine and serum, respectively) using the standard addition methodology [36]. 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 [37] in which a calibration curve is established with continuos variation of sample volume.

By applying linear regression analysis, the slope, the intercept and the regression standard deviation for each curve, (a), (b) and (c) are calculated. The parameters obtained from these three checks are

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

Sample	Spiked[b] (μg ml^{-1})	Found[c] (μg ml^{-1})	Recovery (%)
Human urine-1	5	5.0±0.2	100.0
	10	10.3±0.2	103.0
	15	15.2±0.2	101.3
	20	19.9±0.3	99.5
Human urine-2	5	4.9±0.2	98.0
	10	9.7±0.2	97.0
	15	15.2±0.3	101.3
	20	19.7±0.3	98.5
Human serum	0.40	0.38±0.03	95.0
	0.80	0.77±0.03	96.3
	1.20	1.22±0.03	101.7
	1.60	1.64±0.04	102.5

[a] Using the Student t-test: $R = 101.0\%$; $s_R = 1.84$; $t(R) = 0.208$ ($p = 83.4\%$); critical value, 2.069 (5%) for human urine-1. $R = 98.3\%$; $s_R = 1.97$; $t(R) = 0.438$ ($p = 66.5\%$); critical value, 2.069 (5%) for human urine-2 and $R = 98.9\%$; $s_R = 1.79$; $t(R) = 0.214$ ($p = 83.2\%$); critical value, 2.069 (5%) for human serum.
[b] Referred to original sample.
[c] Average value±S.D. of six determinations.

Table 7
Determination of ofloxacin in human urine samples[a]

Sample	Ofloxacin found[b] (μg ml^{-1})		t	P-value[c] (%)
	Proposed method	HPLC method		
Human urine-1	93.9±0.9	94.2±0.6	2.05	5.7
Human urine-2	95.4±0.8	95.0±0.5	1.96	6.8
Human urine-3	73.1±0.8	73.0±0.6	1.03	31.8

[a] Urinary volume: 1.7 l for human urine-1; 1.6 l for human urine-2 and 2.0 l for human urine-3.
[b] Average value±S.D. of nine determinations.
[c] P value of the comparison test.

reported in Table 4 for urine and Table 5 for serum. From the values in Tables 4 and 5, the OFLX content of the spiked samples is obtained. The results from SC and AC are not significantly different and it can therefore be concluded that the method is accurate. From this study, it can be concluded that the determination of OFLX in human urine and serum samples can be carried out directly by means of SC. However, the Youden's blank should be considered in all measurements for OFLX determination.

Moreover, the validation of the proposed method for urine and serum samples was tested by using a recovery test (Student t-test) [38,39]. Urine and serum samples were fortified with different levels of OFLX. Since the P-values calculated, 83.4% for human urine-1, 66.5% for human urine-2 and 83.2% for human serum, are greater than 5%, so the null hypothesis appears to be valid, i.e. recovery is close to 100% (Table 6).

3.5.2. Real samples

Also, the proposed method was applied to determine OFLX in human urine samples from healthy volunteers who received a single oral dose of 200 mg of ofloxacin. The samples of individuals were collected for up to 24 h after administration of OFLX and the urinary volumes were recorded as well.

In this case, the HPLC with fluorimetric detection method proposed by Macek et al. was used as a reference method [10].

The results obtained, summarised in Table 7, shown that both methods (spectrofluorimetric and chromatographic) yield values within the same range when tested using adequate statistical procedures [40].

4. Conclusions

A sensitive, quick and easy solid-phase spectrofluorimetric method is presented for the determination of ofloxacin in human urine and serum samples (0.5–16.0 ng ml^{-1}), without the need of a pre-separation step. The detection limit obtained, 0.14 ng ml^{-1}, was the lowest reported up to date. It was applied satisfactorily to analyse biological samples with good recovery rates in all cases. The method was validated by comparison with a HPLC method.

Acknowledgements

This work was supported by the Spanish Commission Interministerial of Science and Technology (CICYT) (Project No. PB96-1404).

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