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Spectrofluorimetric and micelle-enhanced spectrofluorimetric determination of gatifloxacin in human urine and serum

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Abstract

A spectrofluorimetric method to determine gatifloxacin has been developed and applied to the quantification of this fluoroquinolone in spiked human urine and serum. The native fluorescence of gatifloxacin allow the determination of 0.040–0.700 μg mL^{-1} of this molecule in aqueous solution containing acetic acid–sodium acetate buffer (pH 3.5), with λ_{exc} = 292 nm and λ_{em} = 484 nm. Micelle-enhanced fluorescence led to 75% higher analytical signals in presence of 12 mM sodium dodecyl sulphate, which allow the determination of 0.020–0.450 μg mL^{-1} fluoroquinolone with λ_{exc} = 292 nm and λ_{em} = 470 nm. Both methods were successfully applied to gatifloxacin determination in spiked human urine and serum.

Keywords: Gatifloxacin; Fluorescence; Micelle-enhanced fluorescence; Urine; Serum

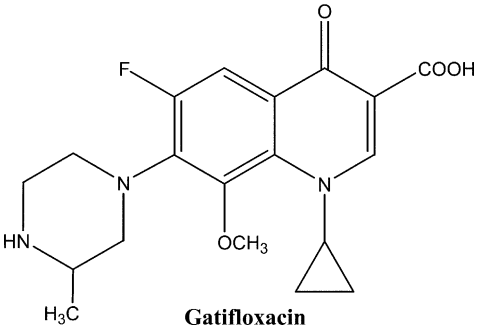
1. Introduction

Gatifloxacin {(±)-1-cyclopropyl-6-fluoro-1,4-dihydro-8-methoxy-7-(3-methyl-1-piperazinyl)-4-oxo-3-quinolinecarboxylic acid sesquihydrate} is a new broad-spectrum 8-methoxyfluoroquinolone antibacterial agent. Gatifloxacin has shown activity against both Gram (+), Gram (−) and anaerobic species, based on the inhibition of both DNA gyrase and topoisomerase IV [1].

Gatifloxacin is supplied to patients with respiratory infections in 200 or 400 mg day^{-1} doses, but it also has been proved to be effective against urinary or cutaneous infections. This fluoroquinolone is mainly excreted in urine (>75%), in unaltered form, and no pharmacokinetic differences have been observed between oral or i.v. administration. Final concentrations achieved in serum and urine of treated patients are 2–5 μg mL^{-1} and approximately 20–100 μg mL^{-1}, respectively [2].

Analytical literature about the determination of this new fluoroquinolone is very scarce. There are a few papers that describe high-performance liquid chromatography (HPLC) methods for the analysis of urine and serum concentrations of this fluoroquinolone using fluorimetric [3], ultraviolet [4,5] and mass-spectrometry [6] detection. There is also a paper [7] that describes a fluorimetric determination of gatifloxacin in urine and serum.

The present paper describes two fluorimetric methods (in aqueous solution and micellar medium) that can be applied as an alternative to the more time-consuming, expensive HPLC methods, with better performance (higher sensitivities and better detection limits) than those reported previously in the literature for fluorimetric determinations.



Gatifloxacin

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2. Experimental

2.1. Reagents

Gatifloxacin was kindly provided by Andromaco S.A. (Madrid, Spain) and Grünenthal (Aachen, Germany). Chemicals of analytical grade were used: concentrated acetic acid, sodium acetate, sodium hydroxide and hydrochloric acid were purchased from Merck (Darmstadt, Germany); sodium dodecyl sulfate (SDS), sodium dodecylbenzensulfonate (NaDDB), Triton® X-100 and hexadecyltrimethylammonium bromide (HDTAB) were purchased from Fluka (Madrid, Spain). High-purity water was obtained from a Millipore (Milford, MA, USA) Milli-Q Plus System. Urine and serum samples were obtained from several healthy volunteers.

Stock standard solution of 200 $\mu g\,mL^{-1}$ was prepared by dissolving accurately measured amounts of gatifloxacin in high-purity water. During the experiments, this solution was found to be stable for several weeks when kept in dark at room temperature. Working standard of 10 $\mu g\,mL^{-1}$ was prepared daily by dilution of stock standard solution with high-purity water. The pH values in optimisation stage were adjusted by the addition of NaOH and HCl until the target pH value was reached.

2.2. Instrumentation

Fluorescence intensity was measured on a Perkin-Elmer (Norwalk, CT, USA) LS-5 luminescence spectrometer equipped with a xenon-lamp and a Acer Model 1030 computer working with the FLUORPACK software from Sciware (Mallorca, Spain). All the measurements took place in a standard 10 mm path-length quartz cell, thermostated at $25.0 \pm 0.1\,^{\circ}C$, with 5 nm bandwidths for the emission and excitation monochromators.

A Philips (Eindoven, Netherlands) Model PU-8720 UV–VIS spectrophotometer was used for the absorbance measurements, and the pH was measured on a Crison (Barcelona, Spain) micropH 2002 pH-meter.

2.3. Sample preparation

The proposed methods were applied to the determination of gatifloxacin in spiked human urine and serum, kindly provided by healthy volunteers.

Urine and serum samples were spiked with convenient amounts of gatifloxacin stock solutions, for final fluoroquinolone concentrations in the range 20–100 and 2.0–5.0 $\mu g\,mL^{-1}$, respectively (media values found in the pharmacokinetic literature). Spiked urine was 25-fold diluted with high-purity water, and no further pre-treatment was required for serum samples.

2.4. Determination of gatifloxacin

For aqueous-solution fluorimetric method, aliquots of working solutions of gatifloxacin, 5 mL of 0.1 M phosphoric acid–sodium phosphate buffer (pH 3.5) were pipetted in to 25 mL calibrated flasks and diluted to the mark with water. Final gatifloxacin concentrations were over the range of 0.030–0.700 $\mu g\,mL^{-1}$. The obtained solutions were thermostated at $25.0 \pm 0.1\,^{\circ}C$ and the fluorescence emission at 484 nm was measured, using an excitation wavelength of 292 nm, against a blank solution.

For micelle-enhanced fluorimetric method, a similar procedure was applied. In this case, 5 mL 0.1 M acetic acid–sodium acetate buffer (pH 5.5) was used, and 2 mL of 0.15 M SDS solution was added before diluting to the mark with water. Final concentrations of gatifloxacin in this case were over the range 0.010–0.450 $\mu g\,mL^{-1}$. These solutions were also thermostated at $25 \pm 0.1\,^{\circ}C$ and the fluorescence was measured at 470 nm, with an excitation wavelength of 292 nm.

For the determination of gatifloxacin in spiked urine and serum, 1 mL of diluted urine or 0.5 mL of serum were put in 25 mL calibrated flasks. The solutions were prepared and their fluorescence was measured following the same procedure as that for standard solutions, with final fluoroquinolone concentration over the ranges 0.030–0.160 and 0.040–0.100 $\mu g\,mL^{-1}$, respectively. In order to avoid the effect caused by the biological matrix in the measurements (as slopes of calibration graphs were found to be 20–30% lower in presence of urine or serum), the standard addition method was applied for the quantification of the fluoroquinolone.

3. Results and discussion

3.1. Preliminary studies and aqueous solution-fluorescence properties

The spectrophotometric properties of gatifloxacin were studied registering the absorption spectra of several 5.0 $\mu g\,mL^{-1}$ gatifloxacin solutions at various pH values. Gatifloxacin showed a maximum absorption at 292 nm at acid pH values, that shifted to 284 nm for higher than 7.0 pH values. Fig. 1 shows the absorbance values of gatifloxacin solutions at different pHs, measured at 292 nm.

With the data obtained from these measurements, and applying the Stenstrom–Goldsmith method [8], a value of pK_a of 6.19 was estimated for this fluoroquinolone. This value is similar to those found by other authors for different fluoroquinolones [9], corresponding to their respective carboxylic groups (Fig. 2). There is a second pK_a value due to the presence of a piperazin group: deprotonation of N4 occurs at pH values greater than 7.0, but the pK_a cannot be accurately calculated with spectrophotometric measurements, as changes in these group does not lead to changes in absorbance properties of the molecule.

Gatifloxacin showed a strong native fluorescence signal at acid pHs, but showed a very weak emission at pH higher than 6.5. Fig. 3A shows the excitation and emission spectra ob-

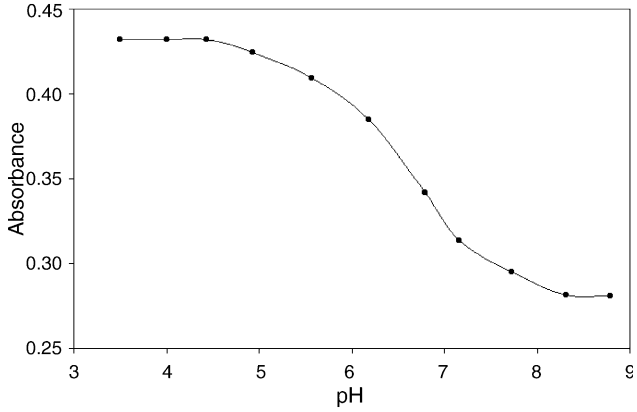

Fig. 1. Effect of pH on the absorbance of 5.0 μg mL^{-1} gatifloxacin solutions at 292 nm.

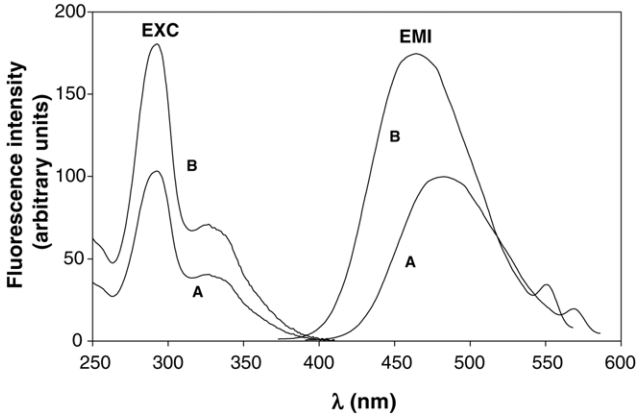

Fig. 3. Excitation and emission spectra of 0.5 μg mL^{-1} gatifloxacin (A) in aqueous medium (pH = 3.5) and (B) in 12 mM SDS micellar medium (pH = 5.5).

tained for a 0.500 μg mL^{-1} aqueous solution of gatifloxacin at pH 4.0. As can be seen, the fluoroquinolone showed an excitation maximum at 292 nm, while the maximum emission is detected at 484 nm. These wavelengths were selected for the following assays to measure the fluorescence intensity in aqueous medium.

Fig. 4A resumes the effect of pH value in the fluorescence signal. Analytical signal exhibits a maximum within a pH range of 3.0–4.0 (this leads us to propose that the fluoroquinolone shows emitting properties in its carboxylic form). Thus, an acetic acid–sodium acetate buffer of pH 3.5 was chosen for future studies. Fluorescence intensity remains stable for a buffer concentration of 5–50 mM, so a 20 mM buffer concentration was selected as suitable for the optimised method.

The effect of the ionic strength was studied by the addition of increasing KCl concentrations to gatifloxacin solutions. The results obtained showed a significant decrease in the analytical signal for concentrations >0.25 M KCl.

Lastly, the temperature influence on the fluorescence intensity was studied, and a nearly linear (negative) relationship between temperature and fluorescence intensity was found. As expected, when the temperature is decreased the fluorescence is enhanced, with a 0.7% °C^{-1} increase. Hence, samples were thermostated at 25.0 ± 0.1 °C (room temperature), as this temperature was easily kept constant.

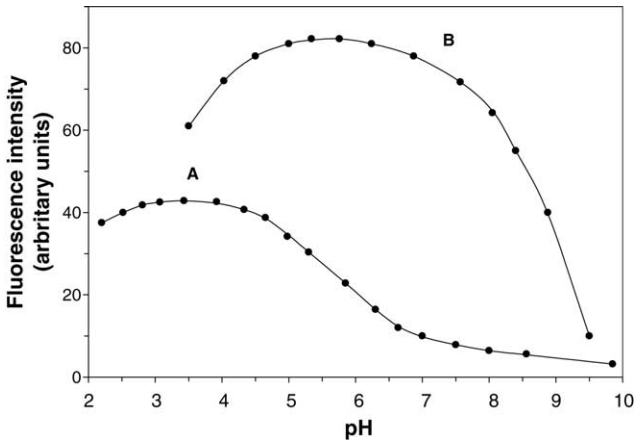

Fig. 4. Effect of pH on fluorescence and micelle-enhanced fluorescence of 0.5 μg mL^{-1} gatifloxacin.

3.2. Micelle-enhanced fluorescence properties

The fluorimetric properties of gatifloxacin were studied in different micellar media, by preparation of differ-

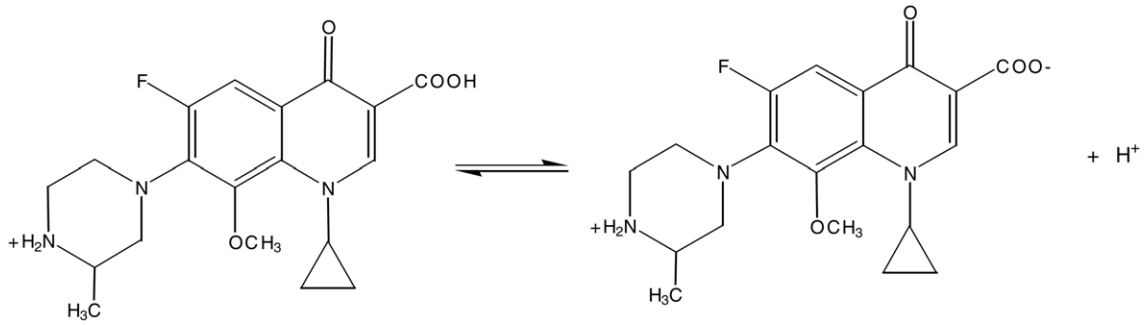

Fig. 2. Protolytic equilibrium of gatifloxacin.

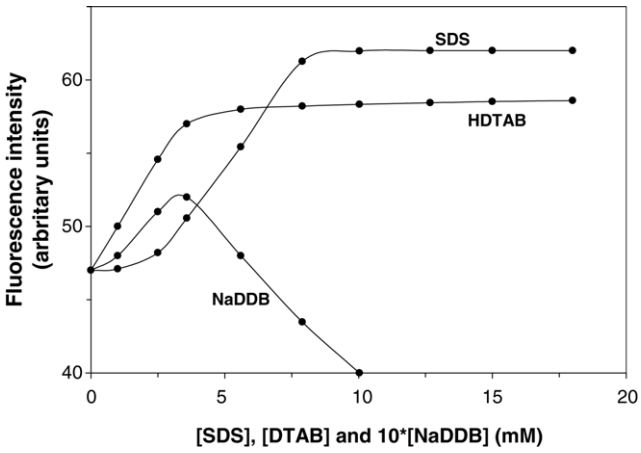

Fig. 5. Effect of SDS, HDTAB and NaDDB addition in the fluorescence emission of 0.5 μg mL^{-1} gatifloxacin (λ_{exc} = 292 nm, λ_{em} = 484 nm; pH = 3.5).

ent 0.500 μg mL^{-1} fluoroquinolone solutions with increasing concentrations of surfactants SDS or HDTAB (cationic), NaDDB (anionic) or Triton® X-100 (non-ionic), at pH 3.5. An increase in fluorescence intensity was observed only when SDS, HDTAB or NaDDB were added. As can be seen in Fig. 5, best results were achieved with SDS solutions with higher than 10 mM tensoactive concentrations, so a 12 mM SDS medium was employed in further studies.

The influence of pH on the micelle-enhanced fluorescence intensity was studied. As can be seen in Fig. 4B, analytical signal reaches a maximum and constant value over the pH range of 5.0–6.5. Thus, an acetic acid–sodium acetate buffer of pH 5.5 was chosen for the micelle-enhanced spectrofluorimetric determination of gatifloxacin. The concentration of buffer showed no influence in analytical signal over the range 5–50 mM, so a 20 mM concentration was selected as suitable for further studies. As can be seen in Fig. 3B, the final fluorescence intensity enhancement achieved was about 75%; λ_{exc} remains unchanged, and a shift can be observed in λ_{em} from 484 to 470 nm. The fact that excitation properties remain unchanged when SDS was added leads to propose that the emitting form of the fluoroquinolone in micellar medium is the same than in aqueous solution (carboxylic form). The shift in optimum pH can be explained as a modification in the acidic properties of the fluoroquinolone when it is dissolved in the interior of the micelles.

3.3. Figures of merit of the fluorimetric and micelle-enhanced fluorimetric determination of gatifloxacin

Two series of 10 standard solutions of gatifloxacin with concentrations between 0.005 and 1.0 μg mL^{-1} were prepared by triplicate and measured by following the procedures described in Section 2. Table 1 resumes the calibration parameters obtained from statistical analysis of the data.

Table 1
Figures of merit of the aqueous solution fluorimetric and micelle-enhanced fluorimetric determination of gatifloxacin

Parameter	Aqueous solution method	Micelle-enhanced method
LRC (μg mL^{-1})	0.040–0.700	0.020–0.450
$a \pm S_a$	0.0 ± 0.6	0.0 ± 0.2
$b \pm S_b$	192 ± 2	336.1 ± 0.7
r	0.9997	0.9998
Linearity (%)	99.0	99.9
Intra-day R.S.D. (%)	1.8	1.0
Inter-day R.S.D. (%)	2.0	1.2
DL (ng mL^{-1})	10	2
QL (ng mL^{-1})	30	6

LRC: linear calibration range; *a*: intercept; *b*: slope; S_a: intercept standard deviation; S_b: slope standard deviation; linearity, 'on-line' linearity; *r*: correlation coefficient; R.S.D.: relative standard deviation; DL: detection limit; QL: quantification limit.

The calibration graph of fluorescence intensity (*Y*) versus gatifloxacin concentration expressed in μg mL^{-1} (*X*) was compared with that obtained by the fluorescence measurement in no micellar medium. As can be seen, regression slope was approximately 75% times higher in the micelle-enhanced fluorimetric method than in aqueous method, confirming the results obtained in the optimisation stage. On-line linearity [10] was found to be 99.0% for fluorescence method and 99.8% for micelle-enhanced fluorescence method.

Detection limit (LOD) and quantification limit (LOQ) were calculated according to the recommendations of the ICH Q2B guide [11] as the analyte concentration giving a signal equal to the blank signal plus 3.3 and 10 times the standard deviations of the blank, respectively. Their values were found to be 10 and 30 ng mL^{-1}, respectively, for the fluorimetric method and 2 and 6 ng mL^{-1} for the micelle-enhanced fluorimetric method. These values were better than those reported previously in literature [7], leading to a 10-times more sensitive determination in the micelle-enhanced fluorimetric method.

In order to study the intra-day precision (repeatability) and inter-day precision (intermediate precision), 11 replicates with a concentration of 0.250 and 0.350 μg mL^{-1} gatifloxacin for the fluorimetric and micelle-enhanced fluorimetric methods were measured (over a 10-day period for the inter-day study). The intra- and inter-day relative standard deviation (R.S.D.) obtained were 1.8 and 2.0% for fluorimetric method and 1.0 and 1.2% for the micelle-enhanced fluorimetric method, respectively.

3.4. Effect of potential interferences

The interference of typical excipients and co-administered drugs (as analgesics or antibiotics) was studied; increasing concentrations of these compounds were added to a 0.250 μg mL^{-1} gatifloxacin solution until a variation in analytical intensity greater than three times the R.S.D. (%) of the method was achieved. Only negative errors were found; maximum tolerable weight ratio for the proposed methods

Table 2
Tolerance of different compounds in the gatifloxacin determination

Compounds added	Maximum tolerance weight ratio	
	Aqueous method	Micelle-enhanced method
Glucose, lactose, fructose, sacarose	2000[a]	2000[a]
Imipenem	1.25	2.5
Paracetamol	10	20
Amikacin	30[a]	30[a]
Aspirin, sulbactam, penicillin	40[a]	40[a]

[a] Maximum weight ratio tested.

are resumed in Table 2. Selectivity achieved by the proposed methods, thus, was good, and made possible to accurately quantify gatifloxacin in presence of the tested compounds.

Gatifloxacin fluorescence was also measured in the presence of some cations typically present in urine and serum samples. There was no interference for Al^{3+}, Ca^{2+}, Na^{+}, K^{+} and Mg^{2+}, Zn^{2+} at molar ratios of cation/gatifloxacin >30. Cu^{2+} and Fe^{3+} interfere negatively at molar ratios over 3.5 and 0.2, respectively (a coloured co-ordination complex was observed when Fe^{3+} and the fluoroquinolone were mixed). Nevertheless, these relations are higher than those typically found in urine and serum samples, so no interference from these cations is expected.

3.5. Determination of gatifloxacin in human urine and serum

The proposed methods were applied to the determination of gatifloxacin in spiked human urine and serum samples, prepared and measured as described in Section 2.

In the literature, different multivariate chemometric procedures have been proposed for separating the fluoroquinolone emission from that of the biological matrix [12–15]. In gatifloxacin case, both urine and serum present minimum emission signals at working wavelengths compared to the fluoroquinolone (present at therapeutic concentrations), as can be seen in Figs. 6 and 7. Nevertheless, both fluids caused quenching effects in gatifloxacin fluorescence; slopes of gatifloxacin calibration graphs were found to be 20–30% lower in presence of urine or serum. So, the standard addition method was applied in order to avoid the influence of the matrix in the results.

Recoveries were calculated for both intra-day (five replicates for each concentration measured in the same day) and inter-day (five replicates for each concentration measured over a period of 10 days). The obtained results for both methods with their respective confidence ranges, calculated as the medium recovery $\pm t \times \text{S.D.}/n^{1/2}$ ($n = 5$, $t = 2.78$ for $P = 5\%$) [16], are resumed in Tables 3 and 4.

The recoveries obtained were compared with the spiked concentration with a Student's t-test [16]. In all cases, the calculated value of $|t|$ was found to be lower than the critical

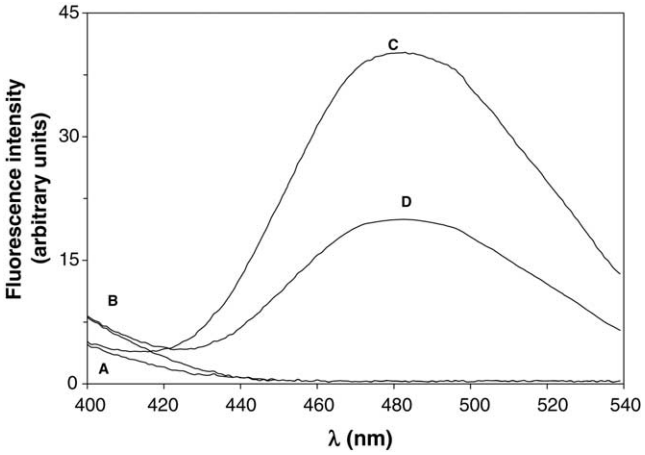

Fig. 6. Emission spectrum in aqueous solution for (A) blank urine sample, (B) blank serum sample, (C) spiked urine sample and (D) spiked serum sample. [Gatifloxacin] = 50 and 2 μg mL^{-1} in urine and serum samples, respectively.

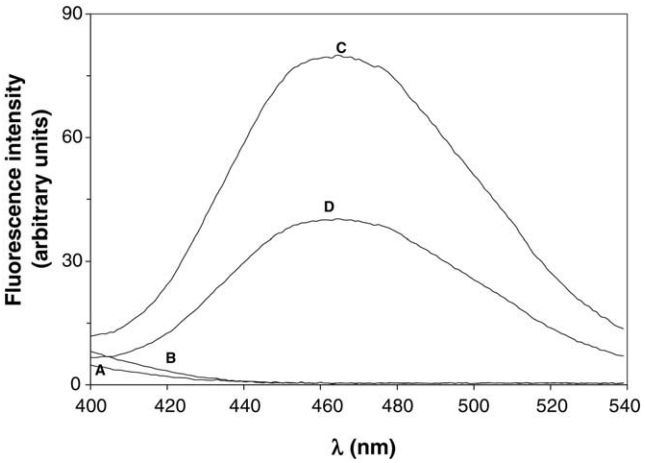

Fig. 7. Emission spectrum in micellar solution for (A) blank urine sample, (B) blank serum sample, (C) spiked urine sample and (D) spiked serum sample. [Gatifloxacin] = 50 and 2 μg mL^{-1} in urine and serum samples, respectively.

Table 3
Intra-day determination of gatifloxacin in human urine and serum

Spiked concentration (μg mL^{-1})	Recoveries[a] (%) ($n = 5$)	
	Aqueous fluorescence	Micelle-enhanced fluorescence
Urine		
20	101 ± 7	97 ± 5
50	101 ± 2	101 ± 2
75	99 ± 1	100 ± 1
100	101 ± 2	102 ± 2
Serum		
2.00	104 ± 7	102 ± 9
3.00	103 ± 5	99 ± 4
4.00	104 ± 5	102 ± 2
5.00	98 ± 2	102 ± 5

[a] Mean $\pm t \times \text{S.D.}/n^{1/2}$ ($n = 5$, $t = 2.78$ for $P = 5\%$).

Table 4
Inter-day determination of gatifloxacin in human urine and serum

Spiked concentration ($\mu g\,mL^{-1}$)	Recoveries[a] (%) ($n = 5$)	
	Aqueous fluorescence	Micelle-enhanced fluorescence
Urine		
20	102 ± 9	101 ± 7
50	102 ± 4	101 ± 2
75	98 ± 2	99 ± 2
100	102 ± 4	98 ± 4
Serum		
2.00	103 ± 10	98 ± 10
3.00	102 ± 6	103 ± 5
4.00	96 ± 7	104 ± 6
5.00	101 ± 5	97 ± 5

[a] Mean $\pm\, t \times$ S.D./$n^{1/2}$ ($n = 5$, $t = 2.78$ for $P = 5\%$).

$|t|$ value (2.78, $P = 5\%$). Thus, there was no difference, statistically, between the experimental recoveries and a 100% recovery.

4. Conclusion

This work shows that fluorimetric and micelle-enhanced fluorimetric proposed methods are simple, accurate, fast and precise procedures to determine gatifloxacin in spiked human urine and serum at levels found after drug administration at normal clinical doses, and may be a viable alternative to traditional HPLC determinations. Micellar SDS medium led to an enhancement in both sensitivity and detection and quantification limits, and the technique was applied successfully for concentration levels 10 times lower than those found in the literature for other fluorimetric determinations.

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